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Prostate cancer is the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery or radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. We use gene therapy, alone and in combination with hormones called cytokines that stimulate the immune system. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT)) is delivered to a cancer cell by a lentivirus so that harmless bacterial proteins are made. When followed by a pro-drug, 5 fluorocytosine (5-FC), cancer cells that make CD/UPRT convert 5-FC to a toxin that kills the original and neighbouring cells. This system works in slow growing tumors like prostate cancer. Killing the tumor cells attracts immune cells. We will identify these and deliver cytokine genes that attract more immune cells into the tumors. We will deliver the cytokine gene alone or with the suicide gene because in other studies, combination therapy works better.

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Table of Contents

Formulated Delivery of Enzyme/Prodrug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

Annual Report, January, 2004. DAMD17-02-1-0107

INTRODUCTION:

Prostate cancer is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatment strategies are needed. The **subject** of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. These genes will be delivered using lentivirus vectors. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5-FC), is then given, cancer cells producing CDUPRT convert 5-FC to a toxin that kills the original cell and others nearby. This system works in slow growing tumors like prostate cancer. Killing the tumor cells attracts immune cells. The **scope** of the work involves preparation of the gene vectors, optimizing the conditions required for delivering the genes of interest using lentivirus vectors, and identification of the immune cells that infiltrate the tumor when gene therapy is used. We will identify these cells and use cytokine genes delivered into the tumor to attract more of them into tumors. We will then compare the effects of delivering the cytokine gene therapy alone, the suicide gene therapy alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of tumor cells that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with prostate cancer.

BODY:

New cell lines have had to be prepared for the studies described below. In addition, we have had to prepare and characterize both plasmids and lentivirus vectors containing the genes of interest for delivery into prostate cancer. Not only have we manufactured the plasmids and recombinant lentiviruses for delivery of the genes of interest, but we have used both to prepare stable transfectants from a prostate cancer line derived from a mouse, the RM1 line, kindly provided by Dr T Thompson, Baylor College, Texas. The use of such cell lines will allow us to generate a maximum effect *in vivo*, and we will then be able to compare the possibilities that can be generated using a lentivirus as the delivery vessel. In the first instance, the work was based on our previous studies that showed that the gene, purine nucleoside phosphorylase (PNP), could be used for gene-directed enzyme prodrug therapy (GDEPT) directed against prostate cancer (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.*, 2002; Voeks *et al.*, 2002). However, due to problems with intellectual property, we were no longer able to use this gene, and moreover, workers from CSIRO decided not to be involved in the ongoing work. Instead, we have recruited Dr Aparajita Khatri, PhD (starting August, 2003) Dr Bing Zhang, PhD (starting November, 2003) and Ms Eboney Doherty, Bsc. Hons, who started in July, 2003. Instead of using PNP, we have chosen the fusion gene, CDUPRT

for the following reasons: CD converts 5 fluorocytosine to 5 fluorouracil, whose metabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) damage DNA and RNA respectively. The rate-limiting step in the generation of 5FdUMP and 5FUTP is the formation of an intermediary metabolite, 5-fluorouridine monophosphate (5FUMP). 5-FUMP is only produced after a series of catalysed enzymatic reactions. This can be circumvented by the ability of UPRT to convert 5FU directly to 5FUMP thereby leading to more efficient production of anti-tumor metabolites, 5FdUMP and 5FUTP (Tiraby *et al.*, 1998). UPRT sensitises cancer cells to low doses of 5FU (Kanai *et al.*, 1998), and when used in conjunction with CD and 5-FC in GDEPT, was more effective than CD-GDEPT alone against colon cancer (Koyama *et al.*, 2000; Chung-Faye *et al.*, 2001) and glioma (Adachi *et al.*, 2000) *in vitro* and *in vivo*. There are no reports of this combination (CDUPRT) being used against prostate cancer, making this application novel. Thus *drugs generated by CDUPRT can kill both dividing and non-dividing cells*. This is important in prostate cancer, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.*, 2000; Pierrefite-Carle *et al.*, 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al.*, 1999).

As we have generated prostate cancer cells transfected with the cytokine genes of interest, we are now in the position to compare the results of directly injecting these transfected cells into the mice, or using lipid-plasmid combinations or lentiviral constructs, which may less efficient, but which would be more likely to have direct application in clinical studies.

DOD Alternate: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. The new program Statement of Work, accepted by the DOD, is shown in Appendix 1. New staff were recruited, as described above, and because of this, the work could not be commenced until July, 2003.

Task 1:
GDEPT alone. Assess the ability of lentivirus expressing GDEPT (based on the fusion gene, cytosine deaminase/uracil phosphoribosyltransferase (CD/UPRT) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 1-12)

a) Prepare recombinant lentivirus
Methods and Results:
Plasmid preparation and characterization: CDUPRT was obtained in the pORF-codA::upp plasmid from Invivogen (San Diego, CA, USA). The CodA::upp(CDUPRT) gene was excised from this plasmid using the *NcoI* and *NheI* restriction enzyme sites and ligated into complementary sites in the pVITRO2-GFP/LacZ expression plasmid (See Appendix 2, Figure 1). pVITRO2-GFP/LacZ, which also contains the genes for the jelly fish green fluorescent protein, GFP, and for the bacterial enzyme, βgalactosidase, LacZ, that can be used as reporter genes to monitor the progress of the preparation of plasmids, was used as a control vector and cell line throughout these experiments and also provided the backbone plasmid for insertion of all genes of interest. The resulting pVITRO2-GFP/CDUPRT plasmid was characterized for the presence of the genes of interest by restriction enzyme digestion using *NcoI* and *NheI* (Appendix II, figure 1).

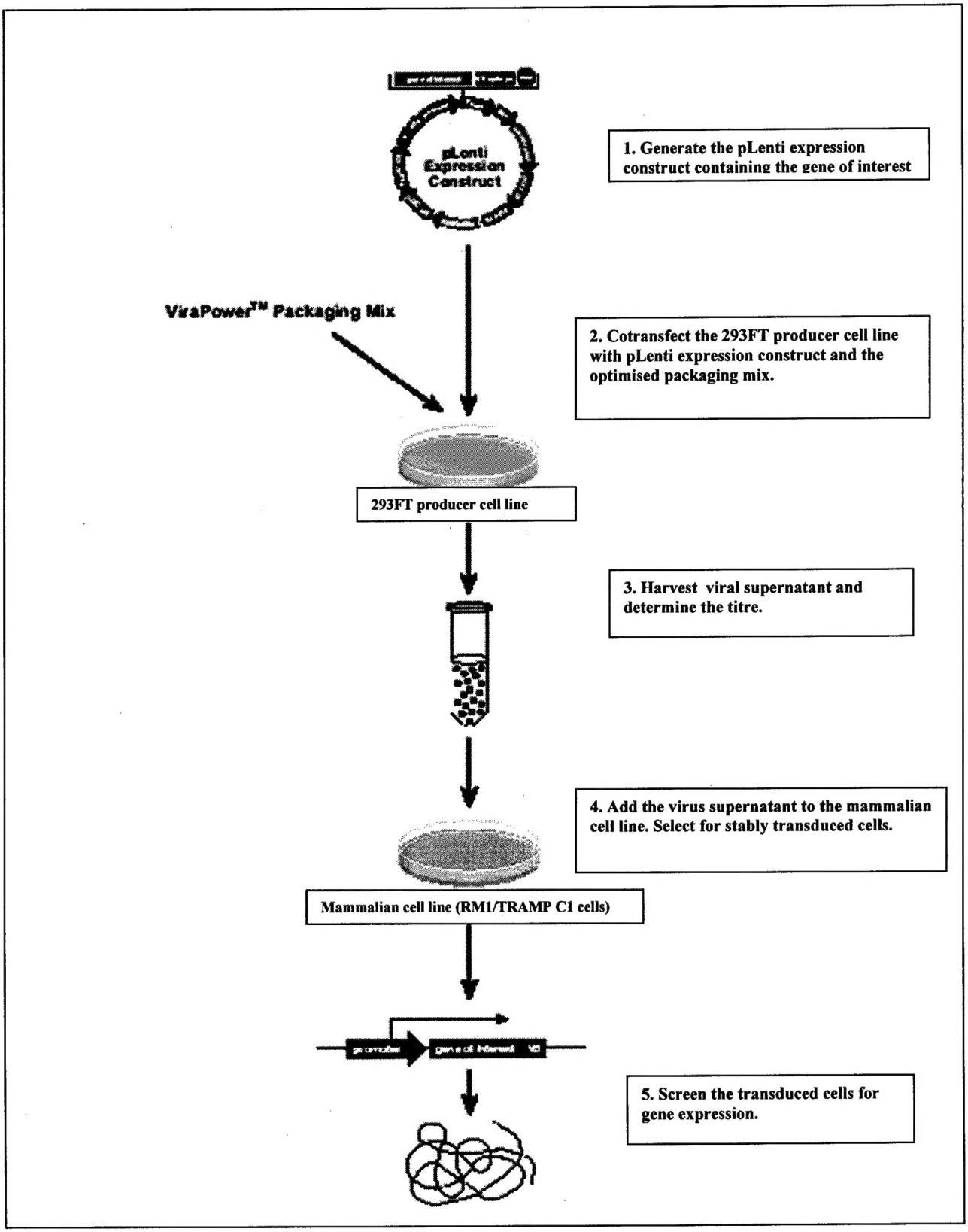
Virus preparation and characterization:

The recombinant lentiviruses to be constructed and used for this study are Lenti.CMV.CDUPRT (GDEPT) and Lenti.CMV.GFP. The GFP expressing virus will serve as a control virus for all the downstream experiments.

The virus construction is being carried out according to the instructions in the ViraPower™ lentiviral expression system kit (Invitrogen, California, USA). The general strategy for the virus construction is as follows:

The pLenti-based expression vector and the packaging mix will be co-transfected together into the 293FT cell line (INVITROGEN) to produce a lentiviral stock. The recombinant lentiviral stock will then be purified by ultracentrifugation and the purified stocks titred for use in the proposed experiments.

A schematic representation of the protocol is as follows (INVITROGEN):



Construction of pLenti.CDUPRT expression construct:

Gateway technology developed by INVITROGEN was used for construction of the expression clone. This technology is based on the capacity of the bacteriophage lambda for site-specific recombination system facilitating the integration of lambda into the *E. Coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). For our purposes, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman *et al.*, 1985).

Recombination occurs between specific attachment (*att*) sites on the interacting DNA molecules. Recombination is conservative (*i.e.* there is no net gain or loss of nucleotides) and requires no DNA synthesis. Strand exchange occurs within a core region that is common to all *att* sites. Briefly, the construction of the transfer vector involves the following steps:

1. Entry clone containing the gene of interest flanked by the *att* sites is constructed.
2. This entry clone is then recombined with the destination vector (pLenti6/V5-DEST; INVITROGEN) using the Clonase enzyme mix (Invitrogen) in an *in vitro* recombination reaction. This would yield the recombinant lenti-expression vector expressing the transgene for generation of the recombinant virus as described above.

Step 1: Construction of the entry vector containing CDUPRT or GFP genes (pENTR1A-CDUPRT; pENTR1A-GFP): The entry vector (pENTR 1A; Invitrogen), especially designed for use in downstream recombination reactions was propagated in DB3,.1 strain of E.Coli.

The CD-UPRT or GFP genes were PCR amplified from pORF-codA::upp (Invivogen;San Diego, CA, USA) and pVITRO2-GFP-LacZ plasmids (Invivogen;San Diego, CA, USA), respectively (Appendix II, figure2A). To ensure the fidelity and integrity of the amplification, *Pfu* TURBO DNA polymerase (Stratagene; California, USA) was used for the PCR amplification. The primers were designed (Appendix IV) such that the CDUPRT or the GFP sequence

1.contained an ATG codon in the context of a KOZAK translation sequence (**G**/A)NNATG**G**) for proper initiation of translation in mammalian cells (Kozak,1987; Kozak, 1990; Kozak, 1991).

2.was in frame with the C-terminal tag after recombination with destination vector.

3.did not contain a stop codon for expressing the CDUPRT protein combined with C-terminal tag (V5) in the destination vector. This would be useful for antibody-based detection of the vector in downstream applications.

Once the genes successfully amplified with the necessary modifications (Appendix II, figure 2A), the fragments were gel purified (Gene clean spin kit; BIO101inc, CA, USA) and digested with restriction endonucleases (*BamH1* and *ECOR1*) followed by another gel purification. These fragments were then ligated to gel purified BAMH1 and ECOR1 digested pENTR vector, using T4 DNA ligase. The ligated product was used to transform chemically competent TOP 10 *E.Coli* strain (Invitrogen), which were then selected under kanamycin selection. The positive clones were screened using the appropriate restriction enzymes for digestion (Appendix II, figure 2B). *We have successfully generated pENTR-CDUPRT and pENTR-GFP.* Integrity of the entry clones will be further confirmed by sequencing. This is currently underway. The primers have been designed (Appendix IV) and clones are being sequenced.

Step 2: Recombination of pENTR-CDUPRT or pENTR-GFP with the destination vector containing the Lentiviral elements (pLenti6/V5-DEST):
The protocol followed for the recombinations are in accordance with the instructions in the supplier's kit manual (Invitrogen). The destination vector, pLenti6-V5DEST (Invitrogen) was propagated in DB3.1 *E.Coli* strain (Invitrogen). Briefly, 300 ng of entry clone was recombined with 300 ng of destination vector in an *in vitro* reaction using LR Clonase enzyme mix (Invitrogen). The reaction was carried out at room temperature for 18 hr. At the end of the incubation the reaction was stopped by ten minute incubation with 4μg of proteinase K/ reaction at 37°C. LR recombination mix (4 μl) was transformed into TOP10 strain of *E.Coli*. The clones were selected under Ampicillin (100 μg/ml) and Blasticidin (50μg/ml) selection. The clones positive for pLenti6/V5-CDUPRT were screened using restriction endonucleases *EcoR1* and *KpnI* (Appendix II, figure 2C). Recombinations were successfully done for the pLenti6/V5GP and the transformed clones are currently being screened. The DNA from the positive clones (pLenti6/V5-CDUPRT) is currently being propagated and will then be used to rescue the recombinant viruses according to the manufacturer's protocol (see above).

b. Implantation of TRAMP-C1 cells subcutaneously (sc) in transgenic TRAMP mice:

The aim of this experiment was to determine the take rate of implanted TRAMP C1 tumour cells in TRAMP (transgenic adenocarcinoma mouse prostate) mice, in order to establish the model for the future experiments as proposed in this study. TRAMP C1 is a murine prostate cell line kindly provided by T. Thompson, Baylor College Texas, from spontaneously arising tumours from the TRAMP mice. Seven TRAMP mice were injected sc with *5 x 10^6 TRAMP C1* cells. This was on the basis of the take rate of TRAMP C1 cells determined in C57Bl/6 animals in this laboratory (Voeks et al., 2002). *Tumour growth wasn't detected until 2 months after implantation.* Tumour samples were harvested and stored at –80°C in OCT or in paraffin at room temperature. Sections of these samples were stained with H&E to assess tumour morphology (Appendix II figure3). Examination of these sections showed that morphology of spontaneous tumours ranged from poor to well differentiated. Figure 3 shows an example of relatively differentiated tumour as shown by the glandular architecture of these tumors. The morphology of the subcutaneous TRAMP C1 tumours in comparison, was consistent with poorly differentiated and invasive tumours, showing loss of glandular architecture (Appendix II, figure 3). This could reflect the fact that the TRAMP C1 cell line is derived from a poorly differentiated tumour.

c. Optimize dose of virus needed to establish GDEPT in orthotopically implanted RM-1 tumors when formulated with plasmid.

Prior to performing this task, and given that we now need to optimize a new GDEPT system involving the use of the CD/UPRT gene and 5-FC, the following experiments have been performed.

i.) *Optimization of dose of 5-FC in vivo, and examination for toxic effects.*
In order to determine the results of GDEPT, it was first necessary to determine the maximum non-toxic dose of prodrug that could be used. Mice were injected with 3 different doses of 5-FC, 150, 300 and 500 mg/kg/mouse, every day for 13 days, given intraperitoneally. The mice were monitored daily for general behaviour and condition. The body weight was measured every second day during the experiment. Toxic effects were monitored by assessing liver and

renal function by analysis of the serum samples from 5-FC treated and control mice. This was done by evaluation of the levels of Urea, Creatinine, Alkaline Phosphotase (ALP), Alanine Amino Transferase (ALT) and Asparate Amino Transferase (AST) in the serum samples (Table 1).

Treatment	Urea (mmol/L)	Creatinine (umol/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Reference	**1.4-5.5**	**18-80**	**35-96**	**17-77**	**54-298**
Control	6.7 ± 0.3	29.3 ± 3.3	54 ± 1.7	32.5 ± 5.7	155 ± 25
150 mg/kg/d	6.8 ± 0.4	20 ± 1.5	67 ± 2.5	27 ± 3.1	92.8 ± 16.5
300 mg/kg/d	6.7 ± 0.3	26.5 ± 0.9	62.3 ± 1.7	18.3 ± 1.8	68.5 ± 13.9
500 mg/kg/d	6.8 ± 0.7	25.3 ± 1.8	55.7 ± 4.9	19 ± 1.5	57.3 ± 11.9

Table1: Biochemical analysis of the serum for assessment of renal and liver functions in 5-FC treated mice. Reference values given are for mice as a whole species and can vary with mouse strain, sampling technique etc. Data presented is Mean ± SEM.

These data show that none of the serum markers was differentially expressed in the test-compared with the control animals. This suggested that there was no detectable toxicity even at the highest dose tested.
H&E stained sections from organs such as liver, heart, spleen and kidney were examined by histology for any changes. No detectable toxicity was observed in any of the organs (Appendix II, figure 4) of the treated animals when compared with untreated control animals.

Assessment of CDUPRT expression: This has been done in two ways, in the first, RM-1 cells have been stably transfected with GFP and CDUPRT so that the duration and extent of CDUPRT expression could be monitored.
ii.) Transfection of RM1 cells: Once the pVITRO2-GFP/CDUPRT plasmid was grown in sufficient quantity, it was used to transfect RM1 mouse prostate cancer cells (obtained from Dr T Thompson, Baylor College, Texas, USA) to generate stable transfectants. RM1 cells were also transfected with pVITRO2-GFP/LacZ to generate a control cell line. RM1 cells were seeded at $4x10^5$ cell/60mm dish. Twenty-four hours later, these cells were transfected following manufacturer's instructions using a complex formed by combining 15µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 5µg plasmid DNA in 1 mL of serum free, antibiotic free Dulbecco's Minimal Essential Medium (DMEM, Invitrogen). Stable clones were selected and maintained under hygromycin selection at 800µg hygromycin B per mL of culture medium. Using a FACs sorter, GFP expression was used to sort the high expressers and to eliminate drug-resistant, non-expressing clones (Appendix 2, figure 5). RM1-GFP/LacZ cells were sorted into high, medium and low GFP expressing populations (Appendix II, figure 6).

The stably transfected cells, called RM1-GFP/CDUPRT, were examined to show that the genes of interest were functional as described below:

Development of *in vitro* CDUPRT-GDEPT assay:

Two quantitative *in vitro* assays were developed using the RM1-GFP/CDUPRT cells.

1. Cell proliferation assay: This is a qualitative and a quantitative assay, which was optimized to examine the stably transfected RM1-GFP/CDUPRT cells for a) gene-expression and b) functionality of the transgene.

   Rationale: Cells expressing a functional CDUPRT fusion gene would not proliferate in the presence of the prodrug 5-FC and the cell proliferation would be inversely proportional to the amounts of the prodrug added.

   Method: Preliminary experiments were done to establish the optimal seeding density of RM1 cells to successfully carry out these experiments. The optimal seeding density was determined to be 10^4 cells/well in a 96 well /plate.

   RM1-GFP/CDUPRT cells were seeded at 10^4 cells /well. RM1-GFP/LacZ cells were used as control cells. Twenty-four hours post seeding, these cells were treated with 5-FC (Invivogen) at nine different concentrations from 0.1-100 μg/ml and then analyzed for viability and proliferation 24 hours after treatment with the drug. The calorimetric assay is based on the cleavage of tetrazolium salt WST-1 (Roche, Sydney, Australia) to Formazan dye by mitochondrial dehydrogenases in viable cells. Briefly, WST-1 was added to the cells and incubated for 48 hours at 37°C in a 5% CO_2 incubator according to the supplier's instructions. The absorbance at was measured at 450nm using "Sunrise Touchscreen" microplate reader (TECAN) after shaking the cells for1 minute.

   Results: The RM1-CDUPRT cells express a functional CDUPRT gene. The cells showed marked reduction in proliferation especially above 3 μg/ml of 5-FC. This effect was not present in the control cells (Appendix II, figure 7).

2. Enzyme activity based on spectrophotometry: This is a modification of the calorimetric assay based on measurement of the enzyme (Cytosine Deaminase, CD) activity in the cell lysates of the transgene expressing cells (Nishiyama *et al.*, 1985). Since the CD and UPRT are fused in our construct and the original fusion cDNA was acquired from a commercial source, measuring the cells for CD activity only was considered to be sufficient for our study. This assay was primarily optimized for use in analyzing the enzyme activity in RM1-GFP/CDUPRT tumors growing in mice. The initial optimization was done using the cultured cells.

   Rationale: CDUPRT expressing cells will have measurable enzyme activity leading to conversion of 5-FC to 5-FU.

   Method: RM1-GFP/CDUPRT cells were seeded in a T75 flask. RM1-GFP/LacZ cells were used as controls. Cells were harvested 24 hours later followed by lysis by repeat freeze thawing. The cells debris was removed by centrifugation and the supernatants were collected. The protein contents of the supernatants were measured using the BCA protein estimation assay kit (Pierce, Rockford, IL, USA) according to the supplier's instructions (Appendix II, Figure 8, insert). 3mM 5-FC (900 μl in PBS) was added to the remaining supernatant and the reaction mix was incubated at 37°C. 50 μl aliquots were collected at various time intervals (1, 4, 20 and 24 h) and the reaction was stopped at each time point by addition of 950μl of 0.1M HCl. Absorbance was measured at 290 and 255nm using the spectrophotometer (UV 1601, SHIMADZU). Concentration of 5-FC was determined as follows:

5-FC(mM)=0.119xA290-0.025xA255.
The enzymatic unit of CD is nmol of 5-FC catalysed per min per mg of protein from whole cell s/n.

Result: The assay was successfully optimized and showed that RM1-GFP/CDUPRT cells have measurable enzyme activity (Appendix II, figure 8). This assay will be useful for analysis of enzyme activity when cells are grown in mice for future *in vivo* experiments. The optimization using the tumors is currently underway (see below).

In vivo expression of transgene

In vivo expression of transgene: *In order to confirm that sufficient transgene is expressed to allow its enzymic activity to be measured,* C57BL/6 mice were injected with 2.5 x 10^6 cells RM1-GFP/CDUPRT cells subcutaneously and the tumors were harvested when they reached the size 10x10mm. The tumors were snap frozen and were stored at –80°C until used for analysis. *These tumors were homogenized and homogenates were assessed for enzymic activity by spectrophotometry as described above for the in vitro assay.* RM1-GFP/LacZ tumors served as the controls for this optimization study. The preliminary data obtained is shown (Appendix II, figure 9). Although, the data clearly showed that in RM1CDUPRT tumours the amount of 5-FC catalysed steadily increased with time, the technique requires further optimization for a more accurate analysis. This is currently underway.

Tasks 1 d, e and f have not yet been attempted. They require completion of section c before they can be performed.

However, we have used parental RM1 tumors not subjected to treatment to perform baseline immune cell determinations by immuno-peroxidase staining with the following MAbs: rat asialoGM1 polyclonal antibody that identifies cells that kill tumors via natural cell mediated cytotoxicity (NCMC); F4/80 that binds to a cell surface glycoprotein on macrophages and their precursors; 53-6.7 that binds CD8 on the surface of CTLs; anti-mouse CD4 found on the surface of helper T cells and anti-mouse CD90 expressed by thymocytes, most peripheral T lymphocytes, some dendritic cells and haemopoietic stem cells, but not by B cells. Some CD90 is also expressed by neuronal cells as well as by epithelial cells and fibroblasts. RM1 prostate tumors were fixed with 37.5mM periodate-75mM lysine-2% paraformaldehyde, pH 7.4 (PLP), OCT mounted and snap frozen. Frozen sections (5μm) were placed on SuperFrost® Plus slides (Art# 041300, Menzel-Glaser, Germany) and were incubated sequentially with 1.5% H_2O_2 (5 mins), avidin block (10 mins), biotin block (10 mins), 3% bovine serum albumin in Tris (5 mins) to block endogenous peroxidase/biotin and non-specific Mab binding. Tissue was incubated with the primary Mab for 1 h at room temperature and secondary Mab for 15 mins followed by a 15 min incubation with peroxidase-conjugated-strepavidin (1:200 dilution). Otherwise, tissue was incubated with primary Mab 1 h and secondary Mab for 30 mins followed by a 30 min incubation with ABC reagent (Vectastain *Elite* ABC System, Vector Laboratories; 1:200 dilution). Sections were developed with 3,3'-diaminobenzidine (DAB) and counterstained with haematoxylin. Mabs (PharMingen) used in immunoperoxidase staining included: biotin rat anti-mouse CD90 [Thy-1.2] (#01012D; 1:400 dilution); biotin rat anti-mouse CD4 [L3T4] (#553649; 1:400 dilution); biotin rat anti-mouse CD8 [Lyt-2] (#01042A; 1:200 dilution); protein A affinity purified rat anti-macrophage marker (hybridoma clone F4/80; Austyn and Gordon 1980) used at a 1:800 dilution; protein A affinity purified rabbit anti-mouse/rat asialoGM1 polyclonal antibody (Cedarlane International, #486-10001; 1:400 dilution). Secondary reagents used included:

biotin conjugated rabbit anti-rat Ig (Vector Laboratories #BA-4001); biotin conjugated goat anti-rabbit Ig (Vector #BO-1000). Stained sections were examined using light microscopy (Leitz Laborlux S; Leica) coupled to a video camera imaging system (Sony Hyper HAD, color CCD-IRIS/RGB, model DXC-151AP) equipped with image analysis software Leica Qwin for PC (Appendix II, Figure 10). From each section, 10 microscopic fields showing the highest positive staining at 40x magnification were selected and the number of positive cells within a fixed 450 x 450 pixel frame (0.1520 mm^2) were counted. The average number of positive cells per high power field (HPF) ± SEM for each section was determined. Although few adaptive immune cells identified by the CD4 or CD8 MAbs were observed, some innate immune cells detected by the anti-asialoGM1 (73±43 cells per mm^2), F4/80 (100±37 cells per mm^2) and anti-CD90 (177±43 cells per mm^2) antibodies infiltrated the untreated RM1 tumors (Appendix II, Figure 10).

As we now have the lentivirus constructs, an assay for CDUPRT activity after delivery in vivo, and a usable dose for the 5-FC, we are in an excellent position to complete Task 1.

Task 2.
pCytokine work: Assess the ability of lipid-enhanced delivery of an murine IL-12 or IL-18 expressing plasmids (pCytokine) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

a. Prepare pVitro2-GFP-Cytokine (murine IL-12 and IL-18) constructs

(i) mIL-12:

Preparation of plasmids: The murine IL-12 gene was obtained from the pORF-mIL-12 plasmid from Invivogen. The mIL-12 gene was excised from this plasmid using the *NcoI* and *NheI* restriction enzyme sites and inserted into the complementary sites in pVITRO2-GFP/LacZ expression plasmid (Appendix II, Figure 11). The resulting plasmid, pVITRO2-GFP/mIL-12, was characterized for the presence of the genes of interest by restriction digestion (Appendix II, figure 11).

Transfection of RM1 cells: To examine the expression of the mIL-12 by the plasmid, it was necessary to transfect RM1 mouse prostate cancer cells. It is important to understand the cell surface cytokine receptor profile of a tumor cell line to be modified with cytokine genes in preparation for cytokine gene therapy. The presence of receptors to cytokine transgenes may elicit undesirable effects such as enhanced tumor cell growth following cytokine gene delivery. To ensure that RM1 cells did not respond to IL-12 stimulation, the presence or absence of IL-12 receptors on RM1 cells was examined. IL12 receptor (IL12R) on the surface of RM1 cells was analysed by flow cytometry using commercial monoclonal antibodies (MAbs): biotin conjugated anti-mouse IL12R (IgG2a) MAb (PharMingen 551973, was used at 0.25ug/tube containing 10^6 cells), biotin conjugated mouse IgG2a isotype matched MAb (PharMingen 553455, was used at the same concentration), and secondary reagent strepavidin phycoerythrin (ST PE). Positive control MAb, PE conjugated mouse anti-mouse NK-1.1 MAb (PharMingen 10295B, was used at 1/100 dilution; 100uL per tube). MIL-12R MAbs showed some non-specific binding to dead cells. These were stained with propidium iodide and excluded from the FACScan analysis (Appendix II, figure 12). Low levels of mIL-12R are present on a murine cytotoxic T cell line CTLL-2 derived from a C57BL/6 mouse. This cell line was propagated with 500pg/mL IL-2 (PharMingen 19211T) and used as a positive control cell line for FACScan staining. Figure 12 (Appendix II) is representative of 3 stainings and shows a low level of IL12R expression on CTLL-2 cells (34.6%) with *no staining of RM1*

cells. There was negligible staining with the mouse IgG2a isotype matched MAb on the homogeneous CTLL-2 (2%) and RM1 (3%) cells.

Stable transfectants of RM1-GFP/IL12 were generated as they could be useful for subsequent work. RM1 cells were seeded at $4x10^5$ cells/60mm dish and after 24h, transfected following manufacturer's instructions using a complex formed by combining 15µl Lipofectamine 2000 (Invitrogen) and 5µg plasmid DNA in 1 mL of serum free, antibiotic free DMEM (as described above). Stable clones were selected and maintained under hygromycin B (800µg/mL) selection.

Expression of transgenes: GFP expression by the clones was used to sort the high expressers and to eliminate drug-resistant non-expressing clones. Low, medium and high GFP expressing populations of cells were selected by FACS sorting (BD FACS VANTAGE XE with VIVA option) (Appendix II, figure 13).
mIL-12 expression is currently being examined by ELISA (BD Pharmingen) and by functional assays using murine CTLL2 cells that proliferate in the presence of mIL-12.

In vivo studies using RM1-GFP/mIL-12 cells: In order to investigate the effect that IL-12 expression alone has on tumor growth and to determine a suitable cell number for future RM1-GFP/mIL-12 implantations, C57BL/6 mice were injected sc with either RM1-GFP/mIL-12 cells or RM1-GFP/LacZ control cells at one of the following cell numbers; 1.5×10^4, 1.5×10^5or 1.5×10^6.Tumor growth was measured on days 4, 7 and 14. Tumor samples were harvested on day 14 and stored embedded in OCT media at –80°C or in paraffin at room temperature.

At cell numbers equal to or above 1.5×10^5 cells, RM1-GFP/mIL-12 cells had both a lower tumor take rate (60-66% compared with 100%) and slower tumor growth than equivalent numbers of RM1-GFP/LacZ cells treated similarly. Furthermore mice implanted with 1.5×10^6 RM1-GFP/mIL-12 cells had a lower tumor take rate (60%) than mice injected with 1.5×10^5 RM1-GFP/mIL-12 cells (66%) (Table 2, see below). The growth rate of tumors formed from 1.5×10^6 RM1-GFP/mIL-12 cells was slower than from tumors formed from 1.5×10^5 RM1-GFP/mIL-12 cells. This is shown by the smaller average tumor volume in the 1.5×10^6 cells group and the lack of detectable tumors in this group at Day 7, whereas the 1.5×10^5 RM1-GFP/mIL-12 cells group and both the 1.5×10^5 cells and 1.5×10^6 cells RM1-GFP/LacZ groups had detectable tumors at this time point. Tumor take rates of the 1.5×10^4 cells group of both cell lines were too low to allow an analysis of growth rates.

Table 2: Take rate of RM1-GFP/mIL12 and RM1-GFP/LacZ cells in C57BL/6 mice.

Cells Implanted	Day 7 Take Rate	Day14 Take Rate
1.5×10^4 RM1-GFP/LacZ	0%	33%
1.5×10^5 RM1-GFP/LacZ	100%	100%
1.5×10^6 RM1-GFP/LacZ	100%	100%
1.5×10^4 RM1-GFP/mIL12	0%	40%
1.5×10^5 RM1-GFP/mIL12	66%	66%
1.5×10^6 RM1-GFP/mIL12	0%	60%

These studies indicate that the RM1-GFP/mIL12 cells are tumorigenic, however, the lower growth rate compared with the RM1-GFP/LacZ suggests that the mIL12 being produced by

the tumor cells may cause inhibition of their tumor growth. This suggested that biologically active mIL12 is being secreted by RM1GFP.mIL12 tumours and that the immune system is implicated in the slower growth rate. Hence, formalin fixed tumour sections were H&E stained and analysed by light microscopy to assess immune cell infiltration of these tumours (Appendix II, Figure 13A). Examination of these sections showed that the control tumours (RM1-GFPLacZ) in general, showed no evidence of immune cell infiltration and resulting necrotic areas. In contrast, the RM1-GFPmIL12 tumors were mostly necrotic and heavily infiltrated with immune cells. The nature of the infiltrating cells will be further analysed by immunostaining with specific antibodies against immune cell populations.These results are promising given our plan to combine immunotherapy and GDEPT in the proposed study.

(ii) mIL-18

Preparation and characterization of plasmids: The murine IL-18 hybrid gene was obtained in the pCEXV3/hybrid IL-18 plasmid from Dr Isao Hara (Kobe University School of Medicine, Japan). The mIL-18 gene was excised from this plasmid using the *EcoRI* restriction enzyme sites and inserted into the *EcoRI* site in the mcs of the pVITRO2-mcs/GFP expression plasmid. The pVITRO2-mcs/GFP plasmid was created by ligating the *NheI-AvrII* fragment from pVITRO2-GFP/LacZ containing the GFP gene into the *NheI* and *AvrII* sites left open by the removal of the corresponding fragment of the pVITRO2-mcs plasmid (Appendix II, figure 14). This was characterized for the presence of the genes of interest by restriction digestion using *EcoRI* and a second digestion using *NheI* and *BamHI* (Appendix II, figure 14). The gene sequence was confirmed by sequencing (SUPAMAC) using primers specific to the mIL-18 sequence (Appendix IV), which were positioned to read through the mIL-18 gene and into the adjacent plasmid sequence.
The sequence is shown in Appendix III.

Transfection of RM1 cells: As for IL-12, in order to examine the expression of the mIL-18 by the plasmid, it was necessary to transfect RM1 mouse prostate cancer cells. Initially, to ensure that RM1 cells did not respond to IL-18 stimulation, the presence or absence of IL-18 receptors on RM1 cells was examined. RM1 cells were analysed for the presence of IL-18 receptors by FACs analysis of immunostained cells in a fashion similar to that described for mIL-12R expression. Antibodies used were goat anti-mouse IL18R MAb (R&D Systems AF856, used undiluted at 20uL per tube containing $5x10^5$ cells/tube), normal goat Ig (R&D Systems BAF108, used undiluted at 20uL per tube) and secondary reagent FITC conjugated donkey anti-goat Ig (Chemicon International AP180F, used at 1/100 dilution and 100uL per tube). Positive control MAb, PE conjugated mouse anti-mouse NK-1.1 MAb (PharMingen 10295B, was used at 1/100 dilution; 100uL per tube). Others have previously shown that approximately 50% of mouse spleen Natural Killer (NK cells) constitutively express IL18R (Hyodo et al., 1999). In the C57BL/6 mouse spleen NK cells may be detected using the NK-1.1 MAb (Koo and Peppard, 1984). In this study, C57BL/6 mouse spleen dual colour stained with NK-1.1 PE and IL18R + anti-goat Ig FITC was used as a positive control. Figure 15 (Appendix II) illustrates 3 stainings and shows that NK-1.1^+ cells constitute 4% of spleen leukocytes when 11.5% of spleen cells express IL18R. Dual colour staining showed ~50% of NK-1.1^+ cells co-expressed IL18R as previously reported. RM1 cells do not express NK-1.1. In the same experiment, RM1 cells showed no staining with IL18R MAb or isotype control MAb. *Thus, the RM1 cells do not express mIL-18 receptors.*

RM1 transfectants (RM1-GFP/mIL18) expressing mIL-18 were generated by transfection of pVitro2-GFPmIL18 in RM1 cells in the same manner as described above for mIL-12. GFP expression in the transformed cells was used as the preliminary indicator of successful

transformation (data not shown). The transformed cells are being further characterised for gene expression using mIL-18 specific ELISA based technique.

KEY RESEARCH ACCOMPLISHMENTS:

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP/mIL-12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.

REPORTABLE OUTCOMES:

- Establishment of new cell lines derived from RM1: RM1-GFP/CDUPRT; RM1-GFP/mIL-12; RM1-GFP/mIL-18.
- Dr Rosetta Martiniello-Wilks has been appointed as a Senior Hospital Scientist at Royal Prince Alfred Hospital to set up a GLP facility for Gene Therapy trials. She was the successful candidate for this position because she was a DOD Trainee-fellow.

CONCLUSIONS:

At this stage of the work, we do not have sufficient data for writing manuscripts, abstracts or patents. However, we have optimized all of the systems and are well placed to obtain data quickly from the next phase of the work.

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APPENDICES:

Appendix I contains new Statement of Work approved by DOD.

Appendix II contains figures 1-15.

Appendix III contains the sequence of the mIL-18 gene as determined by sequence analysis of the positive clones.

Appendix IV contains the sequences of primers designed for various purposes during this study.

APPENDIX I: STATEMENT OF WORK

Task 1. GDEPT alone: Assess the ability of delivery of a lentivirus expressing GDEPT (based on the fusion gene, cytosine deaminase/uracil phosphoribosyltransferase (**CD/UPRT**) to suppress orthotopic and metastatic prostate cancer in RM-1 model. (Months 1-12).

a. Prepare recombinant lentivirus (using vector from Invitrogen).

b. Establish conditions for implanting TRAMP-C1 cells sc in TRAMP mice.

c. Optimize dose of virus needed to establish CD/UPRT GDEPT in orthotopically implanted RM-1 tumors when formulated with lipid and control plasmid.

d. Assess ability of optimal doses of CD/UPRT-GDEPT (and control plasmid) injected intraprostatically into RM-1 tumors together with systemic pro-drug (**5 fluorocytosine, 5-FC**) treatment to suppress local prostate and metastatic (lung) tumor development.

d. Examine other tissues for signs of toxicity that might result from escape of the CD/UPRT GDEPT virus from the site of injection.

e. Identify using immunohistochemistry, the immune cell types infiltrating the prostate tumors.

Task 2. pCytokine work: Assess the ability of lipid-based delivery of an , IL-12 or **IL-18** expressing plasmid (pCytokine) or a **combination of both** to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

a. Prepare pCytosine constructs.

b. Determine dose of pCytosine-construct plasmid DNA (0.1-1.0 μg) which, when formulated with lipid and control virus leads to detectable expression of cytokine in orthotopically implanted RM-1 tumors by:

(i) Harvesting tumor cells, culturing and monitoring cytokine production by Western blot.

(ii) Measuring biological activity of secreted cytokine using a cytotoxic lymphocyte (CTL) bioassay.

(iii) Measuring cytokine mRNA production by RT-PCR.

(iv) Identify using immunohistochemistry the immune cell types infiltrating cytokine secreting tumors

c. Determine impact of administering 5-FC prodrug on immune cell recruitment into tumors following injection of cytokine gene plasmid/lipid/control virus administration

d. Determine the persistence of cytokine production by transfected tumors.

e. Compare ability of transfected pCytokines (optimal dose complexed with lipid and control virus) to suppress orthotopic and metastatic RM-1 prostate tumor growth.

f. Choose optimal cytokine gene system on basis of maximum suppression of tumor growth obtained.

Task 3. Combination therapy: Assess the ability of delivery of a combined virus borne GDEPT and lipid delivered plasmid-borne cytokine gene therapy to suppress orthotopic and metastatic prostate cancer in RM-1 model and in TRAMP mice carrying sc TRAMP-C1 grafts. (Months 22-33)

a. Determine whether pCytokine-enhanced immune activity affects GDEPT.

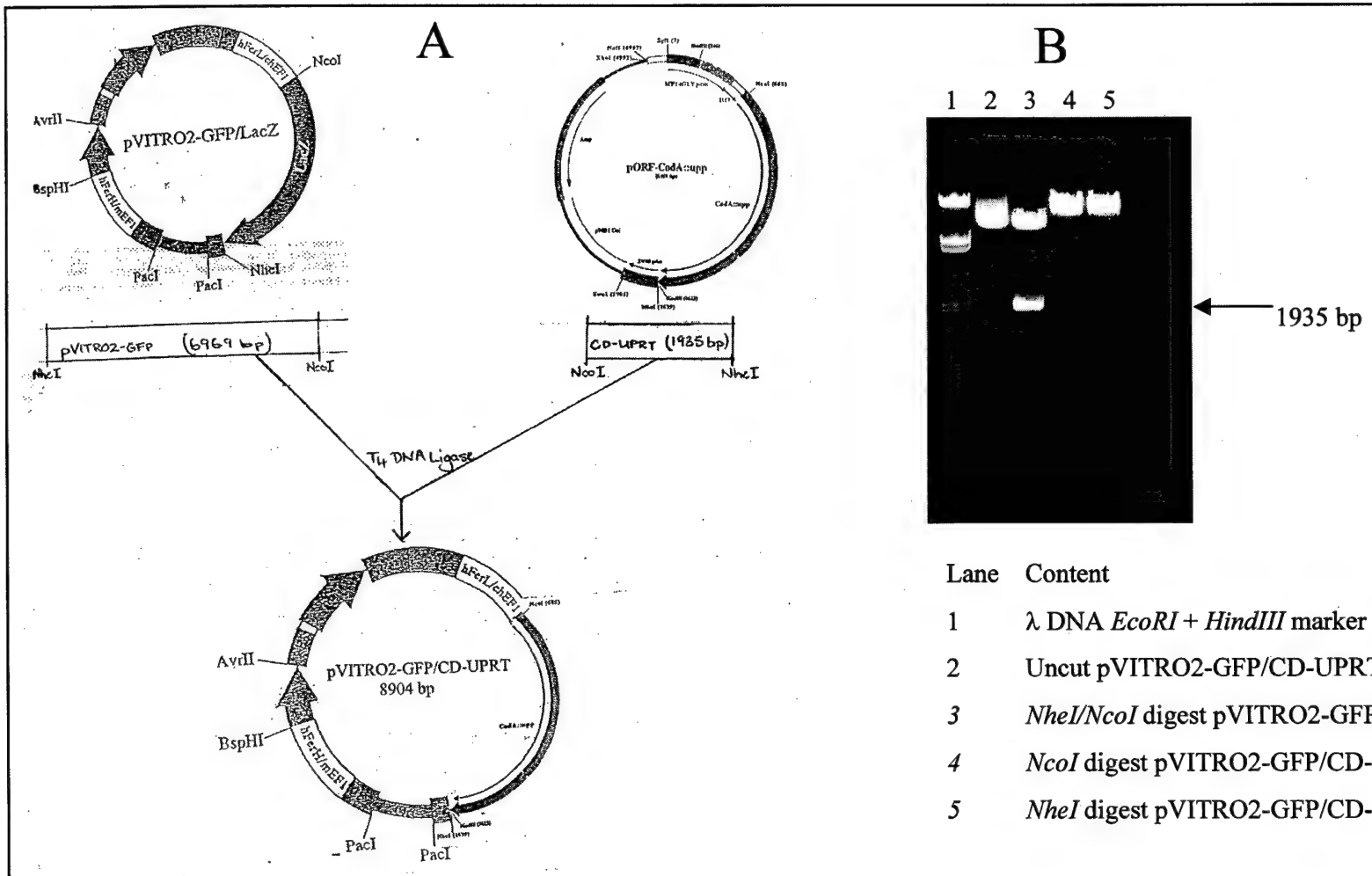
b. Determine the effects of injecting lenti virus expressed GDEPT and pCytokine intraprostatically (using optimal doses of each component as revealed by Tasks 1A and 1B) on orthotopic tumor growth and metastases.

Task 4. Tissue slice work: Assess the ability of OAV encoding green fluorescence protein (GFP) under a prostate directed promoter from Dr Paul Rennie, Vancouver to express GFP in human tissue slices. (Months 24-33)

Task 5. Collate data, prepare reports and manuscripts (Months 33-36)

APPENDIX II

Figures 1-15



Lane	Content
1	λ DNA *EcoRI* + *HindIII* marker (1.5µl)
2	Uncut pVITRO2-GFP/CD-UPRT
3	*NheI/NcoI* digest pVITRO2-GFP/CD-UPRT
4	*NcoI* digest pVITRO2-GFP/CD-UPRT
5	*NheI* digest pVITRO2-GFP/CD-UPRT

Figure 1: Panel A: Strategy for construction of pVITRO2-GFP/CDUPRT. Panel B: Gel image showing restriction digests using *NheI/NcoI* restriction enzymes. The 1935bp fragment (Lane 3) represents the positive clone containing the CDUPRT insert.

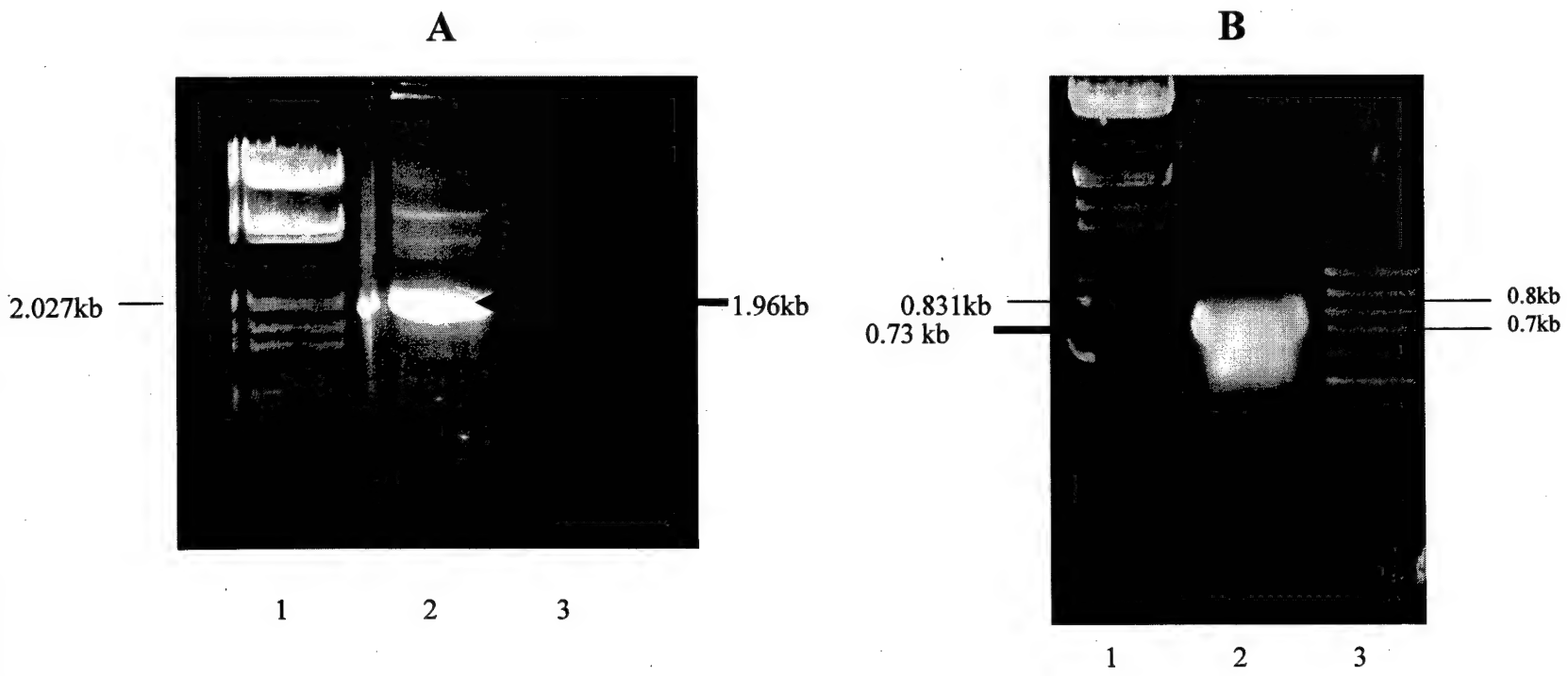


Figure 2A: Agarose gel images showing PCR amplification of CDUPRT (panel A) and GFP (panel B) fragments from pORF-codA::upp and pVITRO2-GFP-LacZ plasmids, respectively. Panel A: Lane 1, λ3 markers (Fermentas);lane 2, PCR amplified CDUPRT(1.965 kb); Lane 3, No DNA control. Panel B: lane 1 and 3, λ 3 and low range markers (Fermentas); Lane 2, PCR amplified GFP (0.73kb).

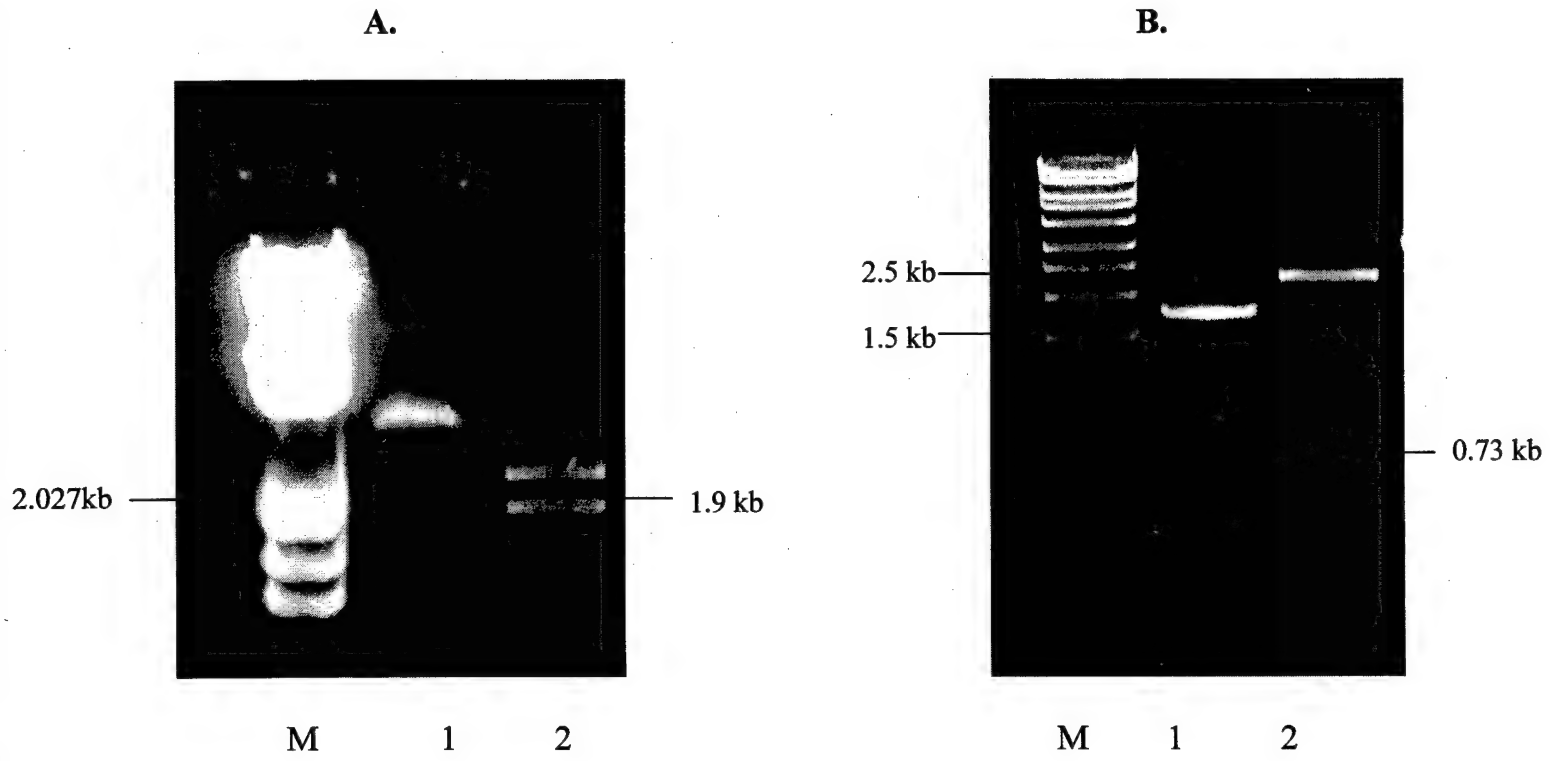


Figure 2B: Gel images representing the restriction enzyme based screening for pENTR-CDUPRT and pENTR-GFP positive clones. 'M' in both gels represent markers (Fermentas).
Panel A: lane 1, uncut DNA; lane 2, a representative positive clone DNA digested with *BamH1* and *EcoR1* restriction endonucleases, 1.9 kb fragment represents the cloned CDUPRT fragment.
Panel B: lane 1, uncut DNA; Lane 2, *BamH1* and *EcoR1* digest of the representative clone generated ~730bp GFP fragment.

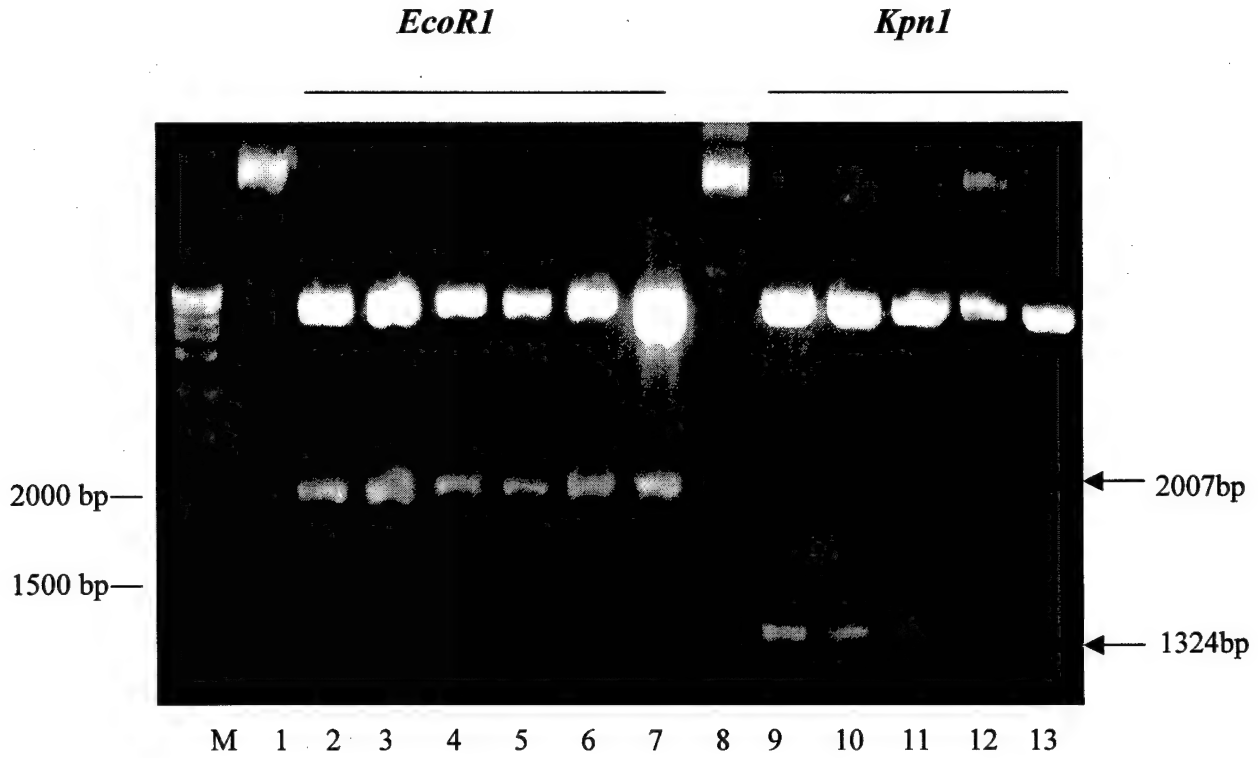


Figure2C: *In vitro* recombination between pENTR.CDUPRT and pLenti6/V5-DEST vectors. Agarose gel image shows the screening for positive clones obtained after transformation of TOP 10 strain of *E.Coli* using the reaction mix produced after *in vitro* recombination between pENTR.CDUPRT and pLenti6/V5-DEST vectors. Restriction endonucleases *EcoR1* (lanes 2-6;Fragment sizes, 2007, 6604) and *Kpn1* (lanes 9-13; Fragment sizes, 1342,7703) were used to screen for the positive clones. M represents the high range markers(fermentas), lanes 1 and 8 show the DNA from uncut representative plasmids. The positive clones contain recombination product, pLenti6/V5-CDUPRT expresion vector.

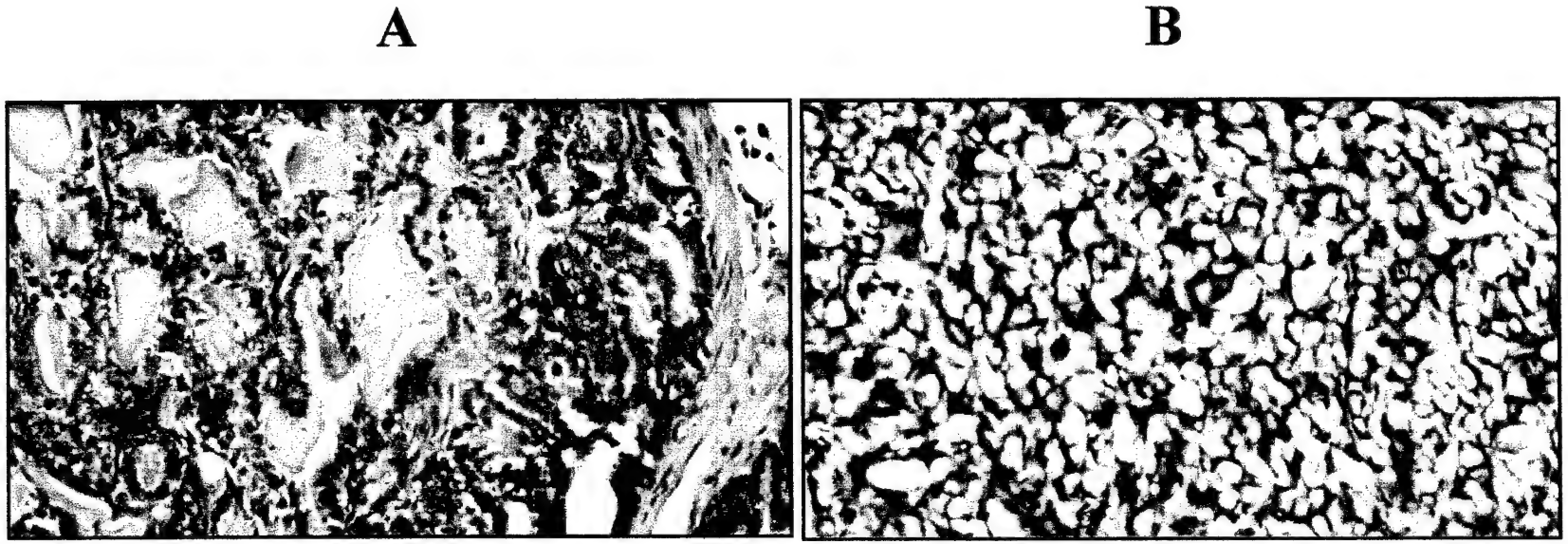


Figure 3:TRAMP C1 Implantation in transgenic TRAMP mice.Panel A: H& E stained section of spontaneous TRAMP tumor from a TRAMP mouse (Magnification 10X) . This is an example of a relatively differentiated tumor as is evident from the glandular architecture. Panel B: H& E stained section of an implanted (sc) tumor using TRAMP C1 cell line in TRAMP mice (Magnification 10X). The implanted tumor has a morphology consistent with a poorly differentiated, invasive TRAMP tumors.

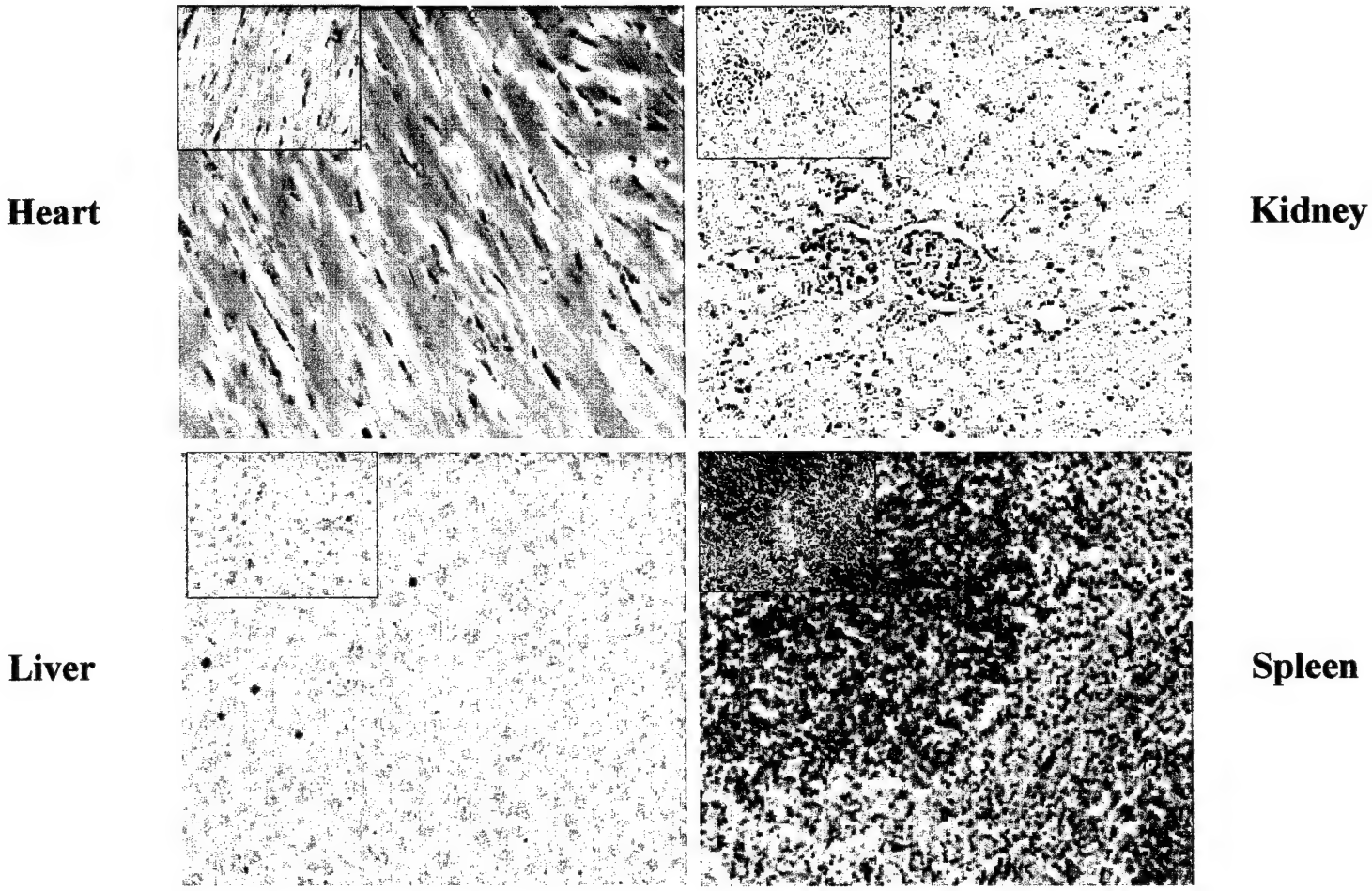


Figure 4: Analysis of toxicity of 5FC in c57bl/6 mice; Mice(4 mice /group) were injected i.p. with 100, 150 and 500 mg/kg of 5-FC for 13 days continuously. A group of four mice received saline instead of 5FC. At the end of the treatment various organs, heart, liver, lung and spleen were harvested and analysed by H&E staining for toxicity effects of the drug. The four panels show different organs from treated mice. The inset in all panels represent the same organ from control mice. There was no detectable toxicity even at the highest dose (500mg/kg) tested.

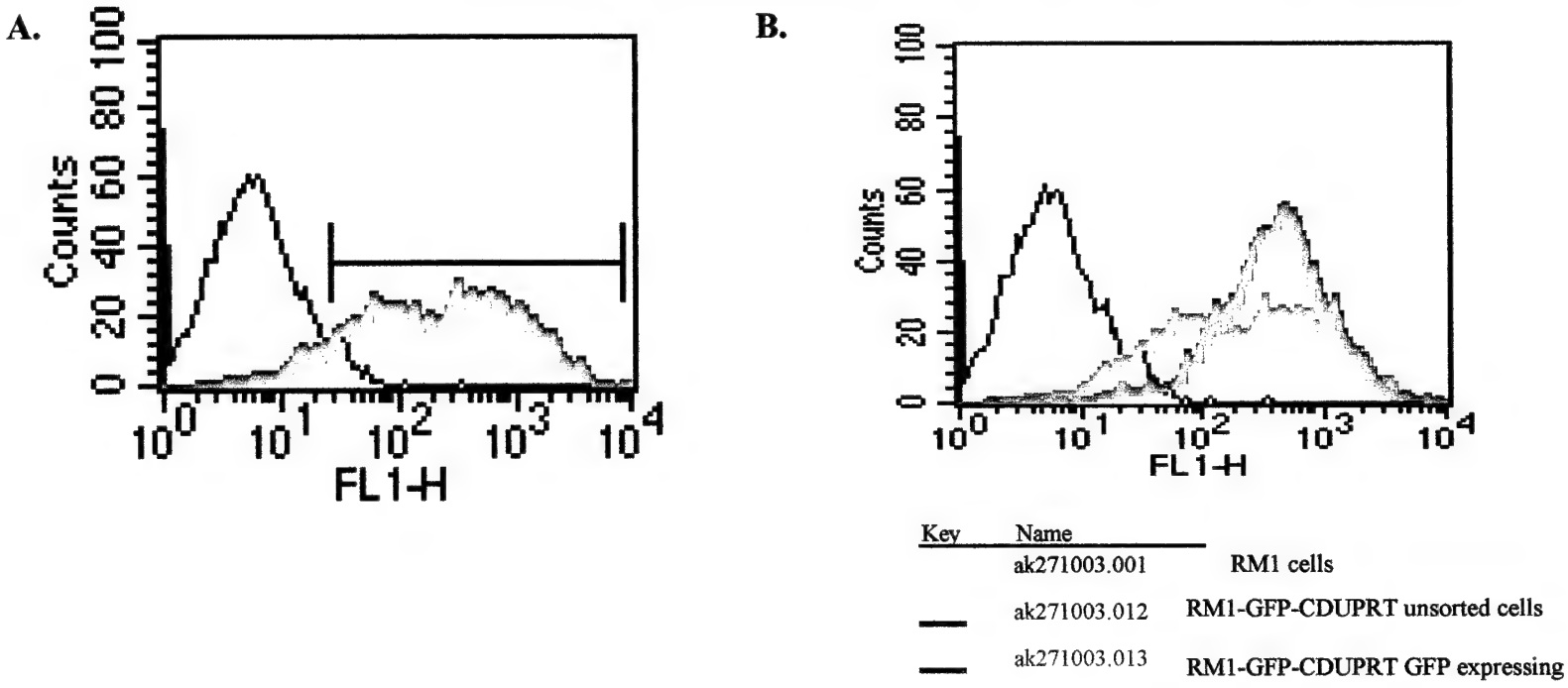
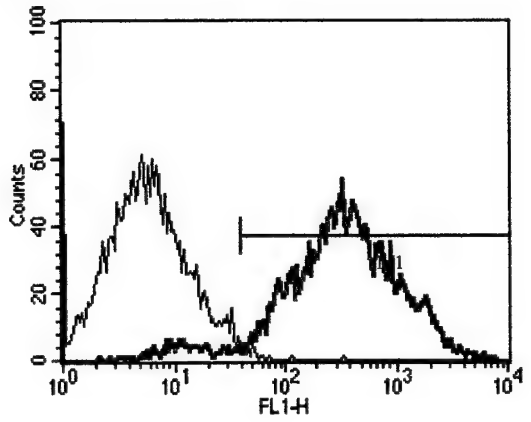
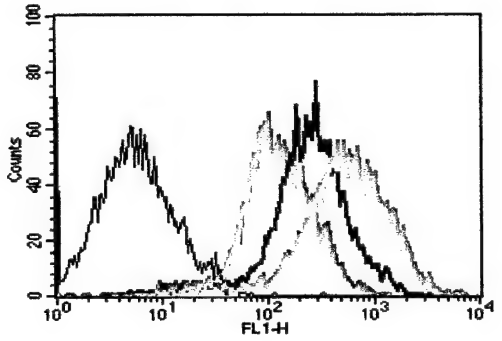


Figure 5: Sorting of RM-1-GFP/CDUPRT cells using a FACS sorter.
Panel A: Unsorted cells , Panel B:Cells sorted into GFP expressing cells.
This was to ensure separation of hygromycin resistant non-GFP expressing cells from GFP expressing cell population.

A.



B.



Key	Name	
	ak271003.001	RM1 cells
	ak271003.003	RM1-GFP-LacZ Low GFP
	ak271003.004	RM1-GFP-LacZ MediumGFP
	ak271003.005	RM1-GFP-LacZ High GFP

Figure 6: Sorting of RM-1-GFP/LacZ cells.
Panel A: Unsorted cells , Panel B:Cells sorted into low, medium and high GFP expressing cells. Cells were separated into three different populations on the basis of level of GFP expression.

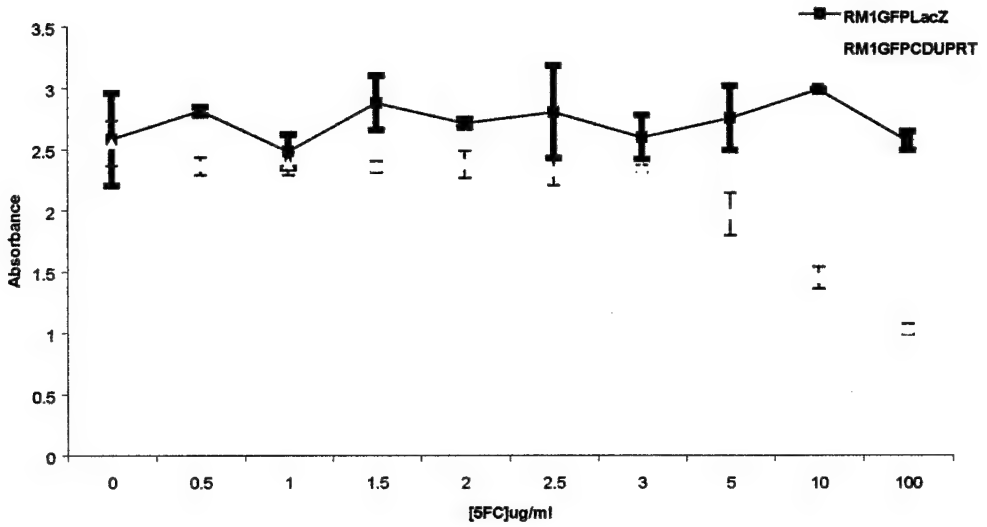


Figure 7: Proliferation of RM-1-GFP-CDUPRT and RM-1-GFP-LacZ cells in response to treatment with different doses of 5FC. RM-1-GFP-CDUPRT cells showed a marked decrease in viability at doses higher than 3 microgram/ml of 5FC.

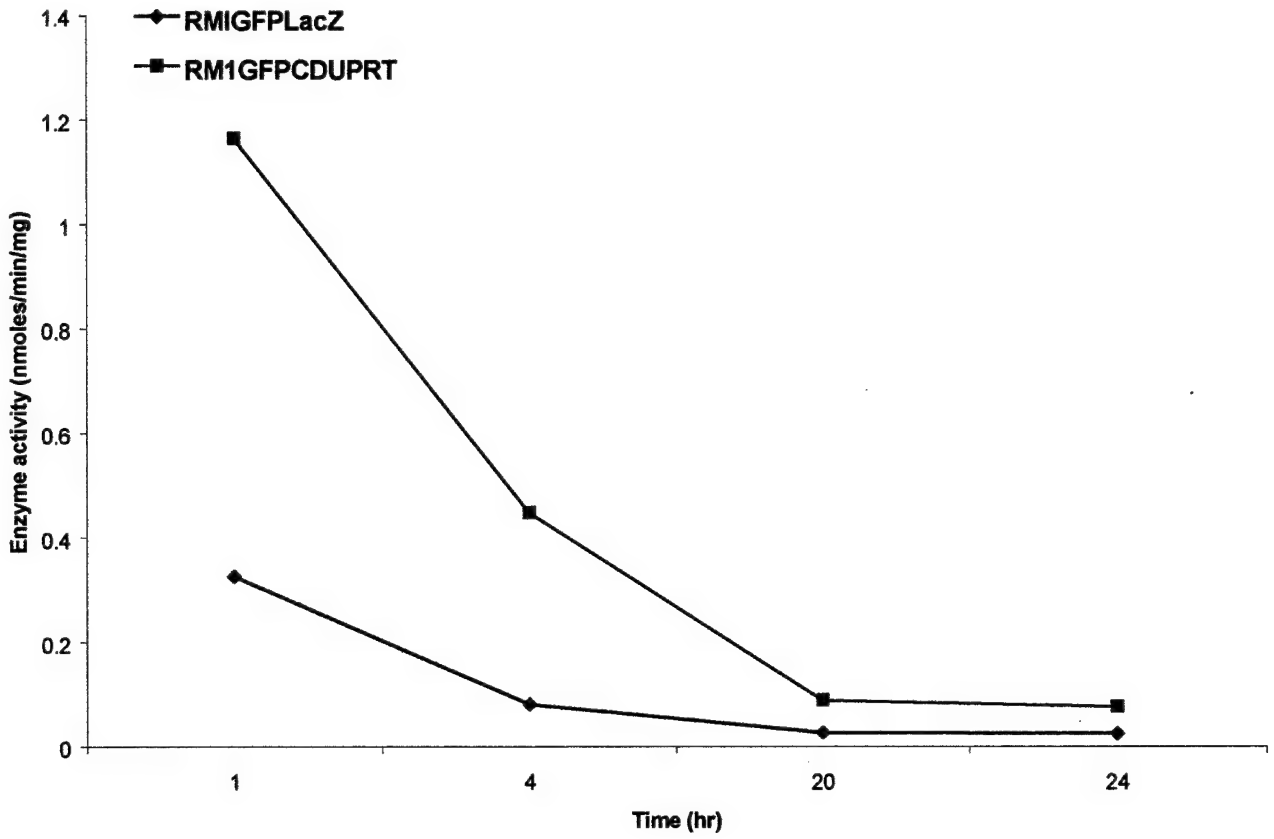


Figure 8: CD-UPRT enzyme activity in RM-1-GFP-CDUPRT cells.
A standard curve generated using the albumin standards was used to calculate the protein concentration of the cell lysates used . The RM-1-GFP-LacZ cells showed some basal non-specific activity.

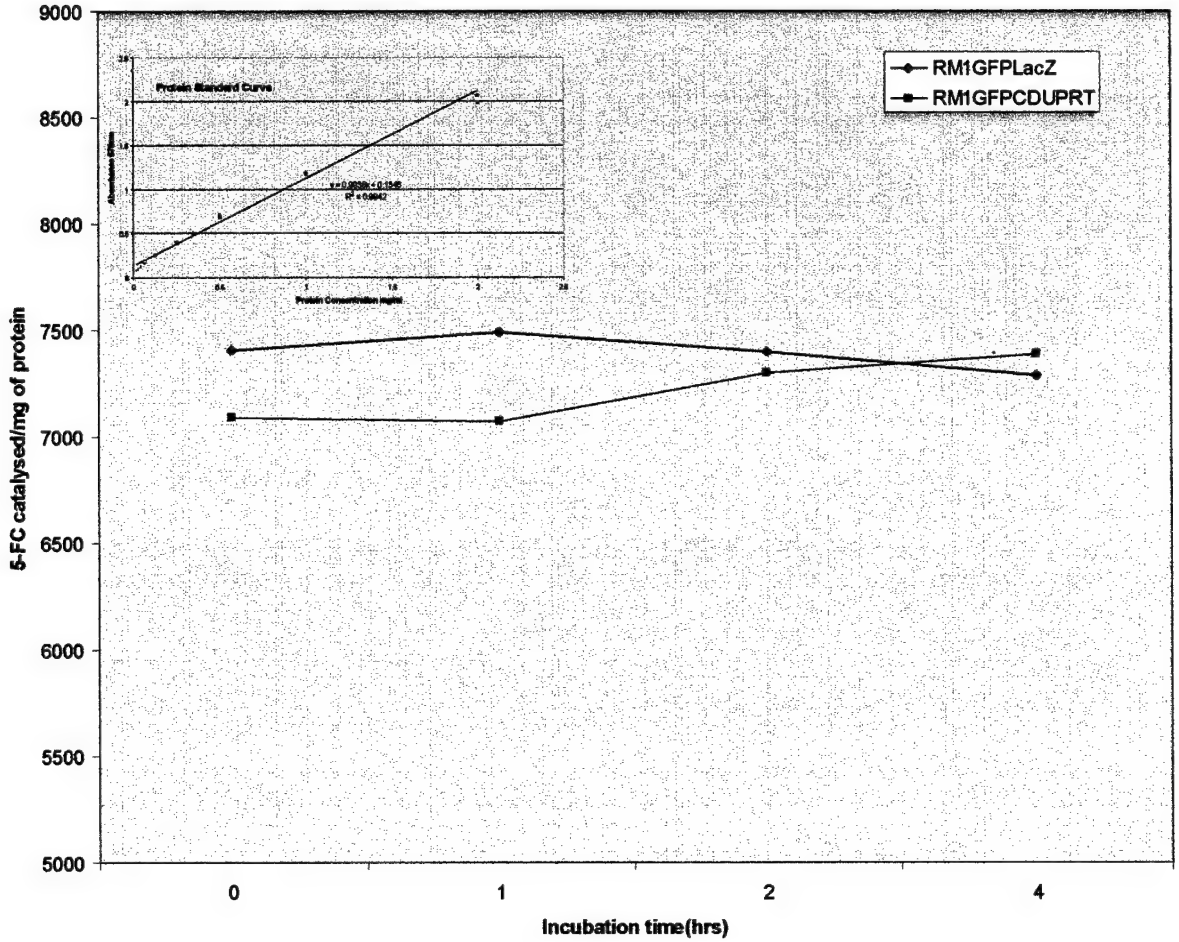


Figure 9: *In vivo* Cytosine deaminase activity. Cytosine deaminase activity was measured by spectrophotometry in RM1GFP-CDUPRT cell derived tumors.

The snap frozen tumors were ground using liquid N_2 and the tumor cell lysates were incubated with 3mM 5-FC for different time periods.The inset shows the standard curve generated using the Albumin standards. This was used to measure the protein content of the tumor lysates. Tumors derived from RM1GFP-LacZ tumors served as controls. Quantity of 5-FC catalyzed gradually increased with time in RM1CDUPRT tumors while it stayed the same in the control samples.

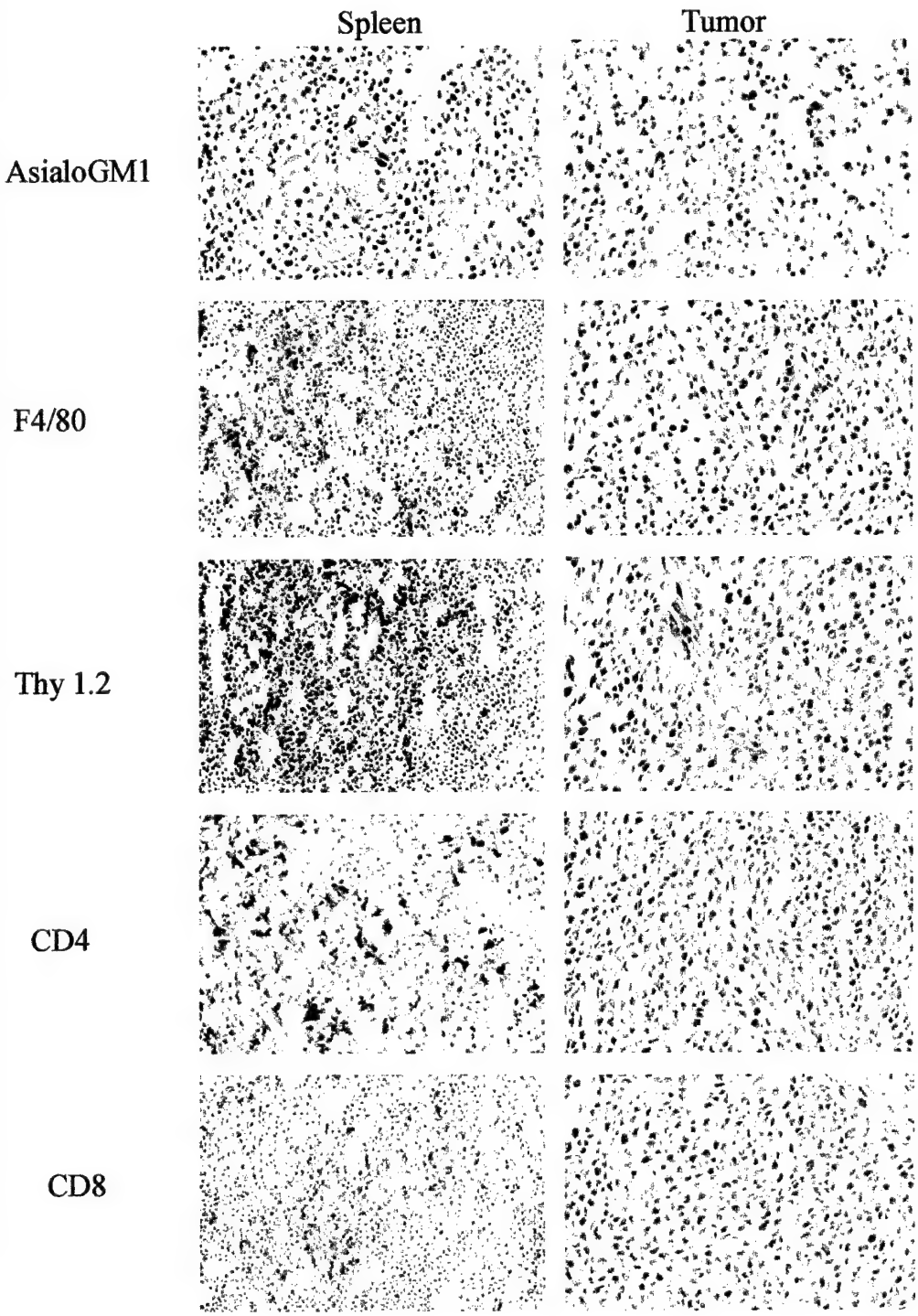
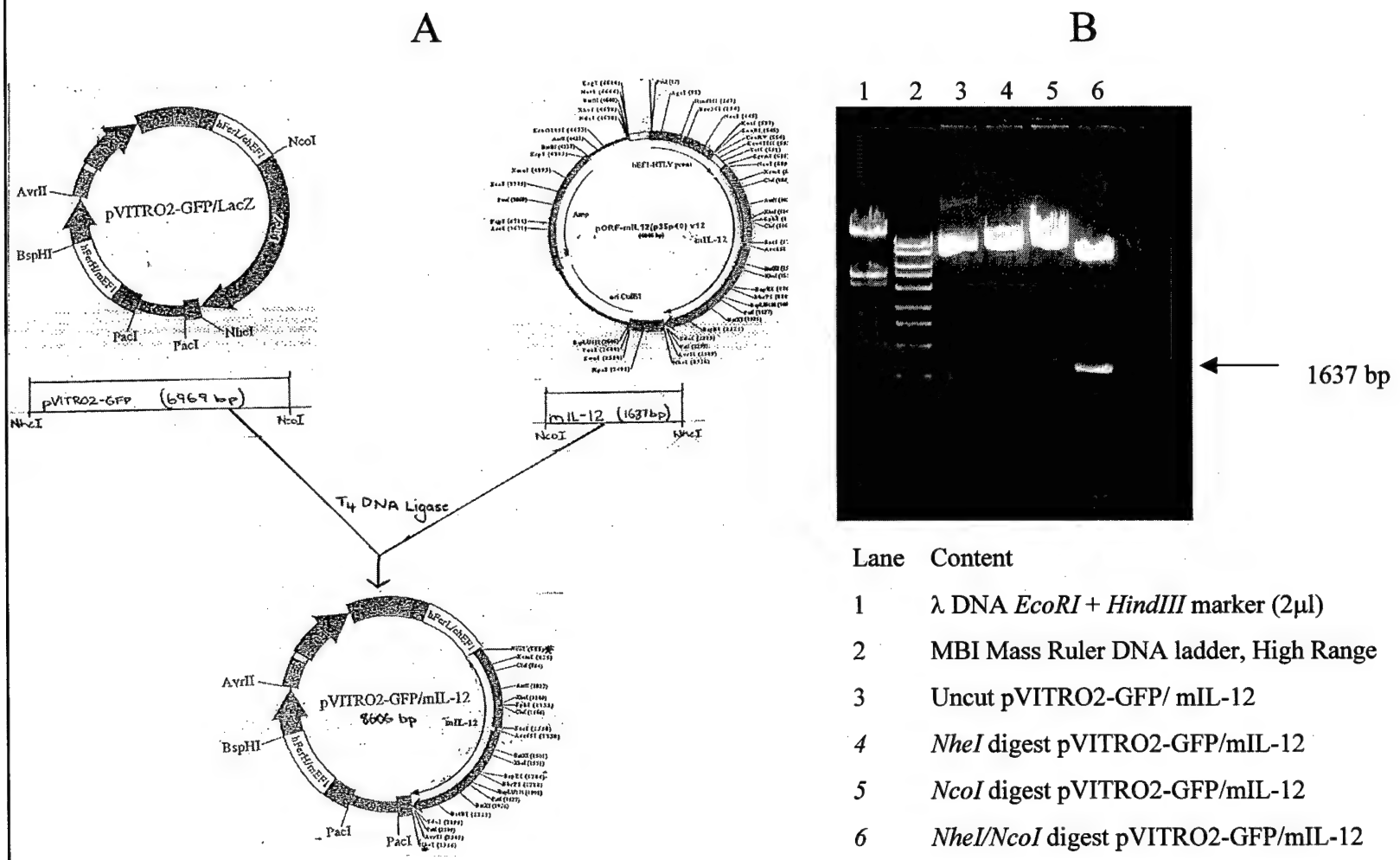


Figure 10: Immunoperoxidase (IPX) staining for lymphocyte subsets in non-treated spleen and RM1 prostate tumor. Magnification 40×.



Lane	Content
1	λ DNA *EcoRI* + *HindIII* marker (2µl)
2	MBI Mass Ruler DNA ladder, High Range
3	Uncut pVITRO2-GFP/ mIL-12
4	*NheI* digest pVITRO2-GFP/mIL-12
5	*NcoI* digest pVITRO2-GFP/mIL-12
6	*NheI/NcoI* digest pVITRO2-GFP/mIL-12

Figure 11. Panel A: Strategy for construction of pVITRO2-GFP/mIL-12; Panel B: Gel image representing *NheI/NcoI* digestion to screen for positive clone. The 1637bp fragment (Lane 6) represents the mIL-12 insert.

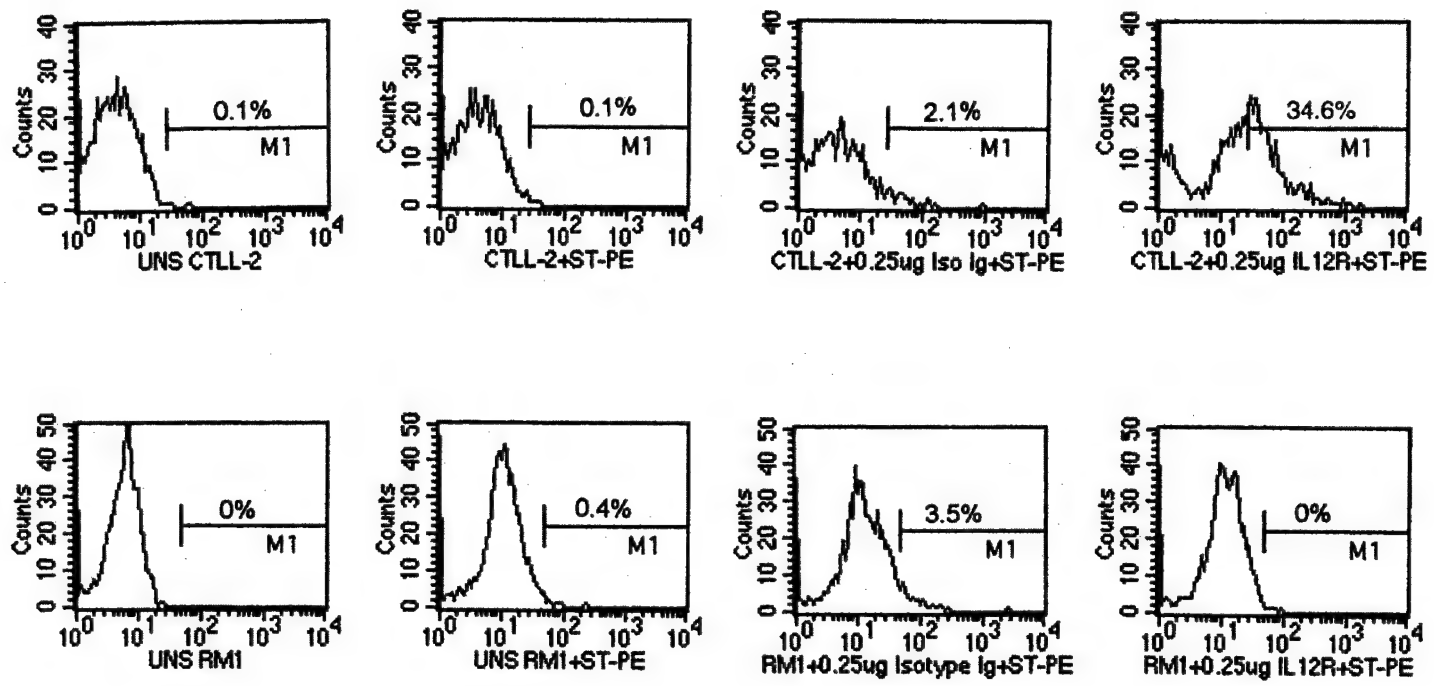
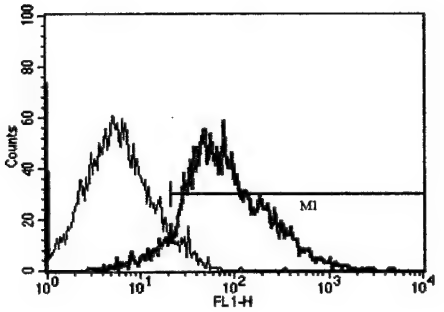
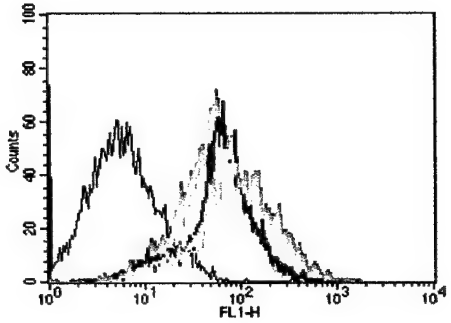


Figure 12: Lack of IL12R expression on RM1 prostate cancer cells. RM1 or CTLL-2 cells (10^6 cells per tube) were stained with 0.25μg biotin conjugated anti-mouse IL12R (IgG2a) MAb (0.25μg IL12R) or biotin conjugated mouse IgG2a isotype matched MAb (PharMingen 0.25μg Isotype Ig) and secondary reagent strepavidin phycoerythrin (ST PE). Dead cells were stained with propidium iodide (PI) and excluded from the FACScan analysis.

A.



B.



Key	Name	
	ak271003.001	RM1 cells
—	ak271003.007	RM1-IL12 low GFP
—	ak271003.008	RM1-IL12 medium GFP
—	ak271003.009	RM1-IL12 lhigh GFP

Figure 13: Sorting of RM-1-GFP/mIL12 cells..
Panel A: Unsorted cells , Panel B:Cells sorted into low, medium and high expressing cells.
Cells were separated into three different populations on the basis of level of GFP expression.

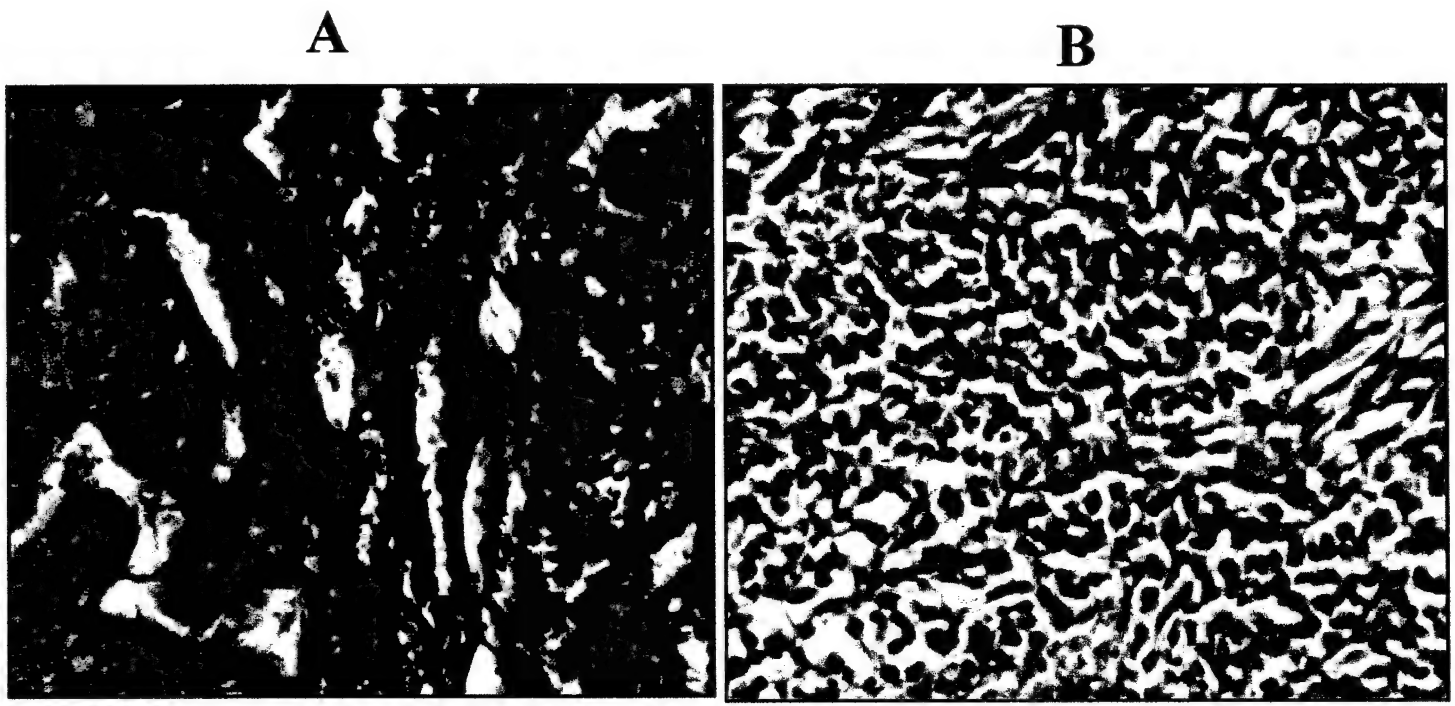
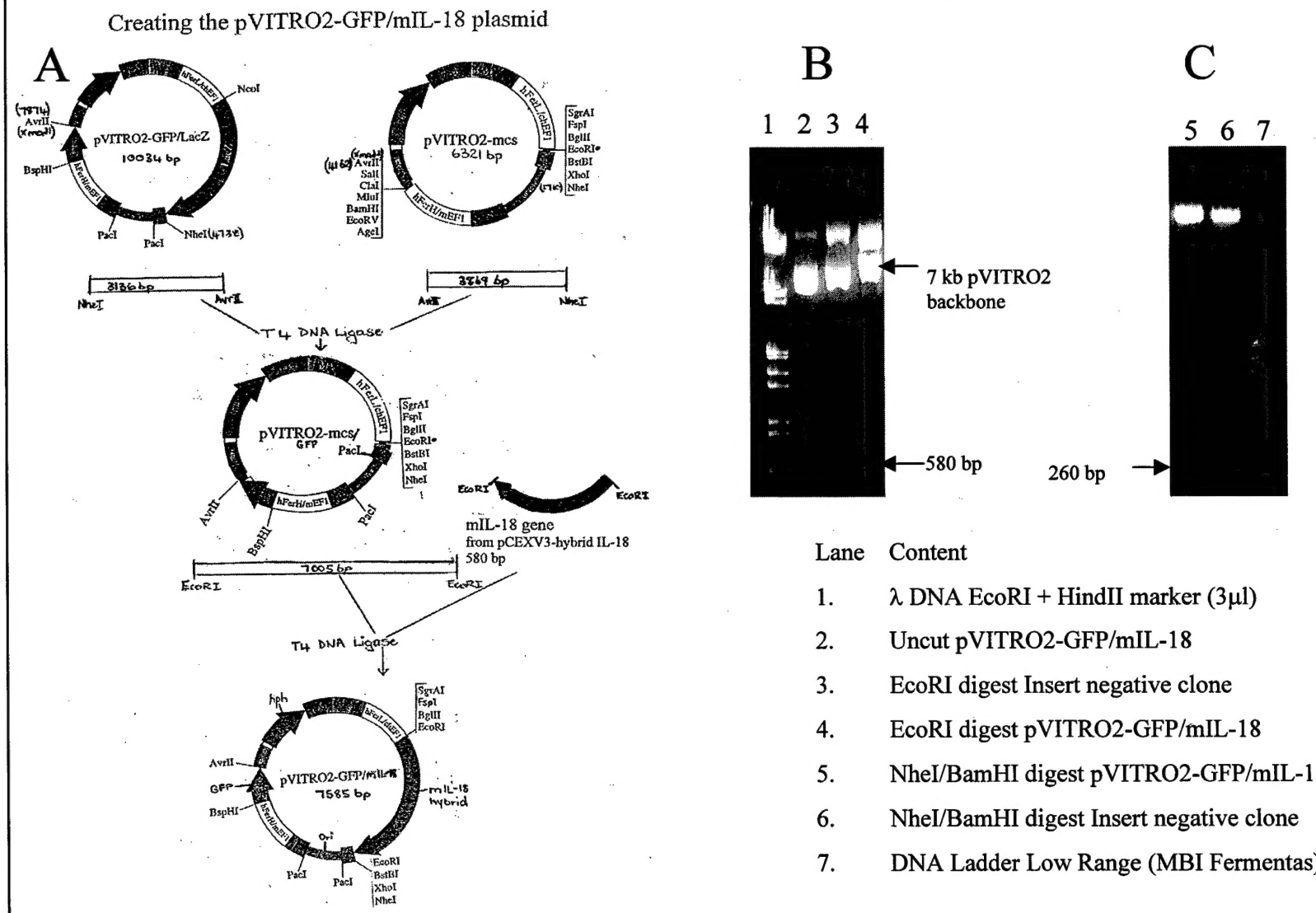


Figure 13A: Immune cell infiltration of RM1-GFPmIL12 tumors *in vivo*. Panel A: H&E stained sections of RM1-GFPmIL12 tumors were highly necrotic and showed evidence of immune cell infiltration. Panel B: H&E stained sections of RM1-GFPLacZ tumors were very vascular and showed no evidence of necrosis due to immune cell infiltration. Both the images are shown at the magnification 10X.



Lane	Content
1.	λ DNA EcoRI + HindII marker (3µl)
2.	Uncut pVITRO2-GFP/mIL-18
3.	EcoRI digest Insert negative clone
4.	EcoRI digest pVITRO2-GFP/mIL-18
5.	NheI/BamHI digest pVITRO2-GFP/mIL-18
6.	NheI/BamHI digest Insert negative clone
7.	DNA Ladder Low Range (MBI Fermentas)

Figure 14: Panel A: Strategy for construction of pVITRO2-GFP-mIL-18; Panels B and C are gel images that represent restriction digests using *EcoI* and *NheI/BamHI* respectively to screen for a positive clone. In Panel B, the 580bp fragment represents the entire mIL-18 insert, and in panel C, the 260 bp fragment (Lane 5) represents the correct orientation of the insert.

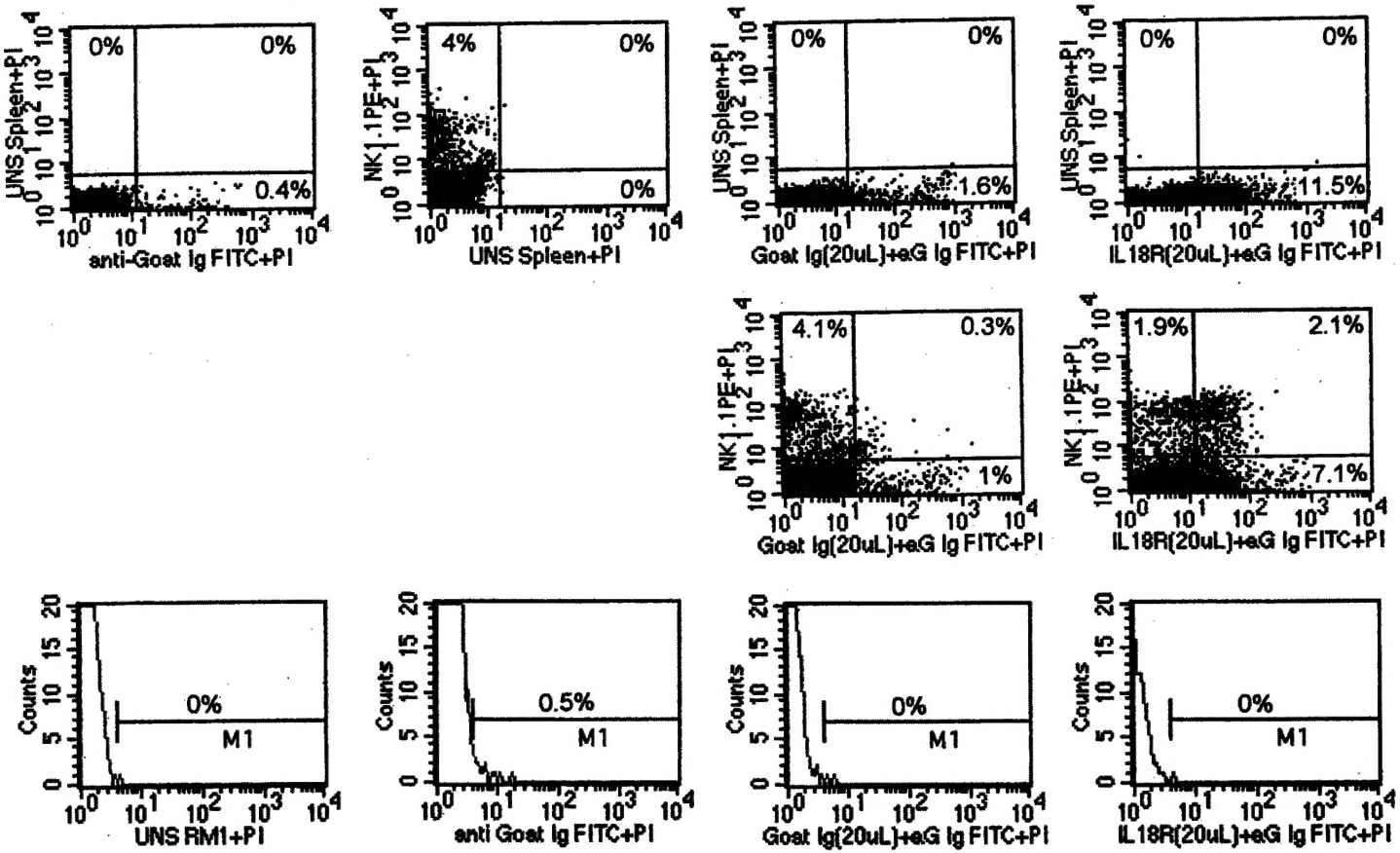


Figure 15: Lack of IL18R expression on RM1 prostate cancer cells. RM1 or C57BL/6 mouse spleen cells ($5x10^5$ cells per tube) were stained with goat anti-mouse IL18R MAb (IL18R 20μL) or normal goat Ig (Goat Ig 20μL) and secondary reagent FITC conjugated donkey anti-goat Ig (aG Ig FITC). Positive control MAb, PE conjugated mouse anti-mouse NK-1.1 MAb (NK1.1PE) was also used for mouse spleen satining. RM1 cells do not express NK1.1. Dead cells were stained with propidium iodide (PI) and excluded from the FACScan analysis.

APPENDIX III:

Sequence of Hybrid mIL-18 gene in pVITRO2-GFP/mIL-18

TTTTNGGGCCGCGGGGGCGACGGGNCCNTGCGTCCCAGCGCACATGTTCG
GCGAGGCGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCT
CAAACTGGCCGGCCTGCTCTGTGCCTGGCCTCGCGCCGCCGTGTATCGCC
CCGCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGA
AAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACGC
GGCGCCCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCC
TTTCCTTCCTCATCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCC
GTCCAGGCACCTCGATTAGTTGTCGAGCTTTTGGAGTACGTCGTCTTTAG
GTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTG
GAGACTGAAGAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAA
TTTGCCCTTTTTGAGTTTGGATCTTGCCTCATTCTCAAGCCTCAGACAGT
GGTTCAAAGTTTTTTTCTTCCATTTCAGGTGTCGTGAAAACTACCCCTAA
CCGGCGTGCGCAAGATCTGAATTCGGCTTACACCAGCCTGGCTTCCATCA
TGAACAACAGGTGGATCCTCCACGCTGCGTTCCTGCTGTGCTTCTCCACC
ACAGCCCTCTCCAACTTTGGCCGACTTCACTGTACAACCGCAGTAATACG
GAATATAAATGACCAAGTTCTCTTCGTTGACAAAAGACAGCCTGTGTTCG
AGGATATGACTGATATTGATCAAAGTGCCAGTGAACCCCAGACCAGACTG
TAATATACATGTACAAAGACAGTGAAGTAAGAGGACTGGCTGTGACCCTC
TCTGTGAAGGATAGTAAAATGTCTACCCTCTCCTGTAAGAACAAGATCAT
TTCCTTTGAGGAAATGGATCCACCTGAAAATATTGATGATATACAAAGTG
ATCTCATATTCTTCAGAAACGTGTTCCAGGACACAACAAGATGGAGTTTG
AATCTTCACTGTATGAAGGACACTTTCTTGCTTGCCAAAAGGAAGATGAT
GCTTTCAAACTCATTCTGAAAAAAAAGGATGAAAATGGGGATAAATCTGT
AATGTTCACTCTCACTAACTTACATCAAAGT**TAG**GTGGGGAGG.

APPENDIX IV:

Sequences of Primers designed for this study thus far:

Primers designed forPCR-amplification of transgenes, CDUPRT and GFP from pORF-codA::upp (Invivogen;San Diego, CA, USA) and pVITRO2-GFP-LacZ plasmids (Invivogen;San Diego, CA, USA), respectively:

The forward 5' primer introduces a *BAMH1*(underlined) site in frame with the coding region and the Kozak sequences are incorporated prior to ATG (start codon).

FWCU5'primer: 5' CCGGCGAAGGGGATCCACC**ATG**GTGTCGAATAACGCT 3' (CDUPRT specific)
FWGFP5'primer: 5'CCGCTAAGGATCCGCAATC**ATG**AGCAAGGGAGAAGA 3' (GFP specific)

The reverse 3' primer is designed such that it eliminates the stop codon (TAA) and introduces *ECOR1*(underlined) as an additional site.

RevCU3'primer: 5' TTCGACAGAATTCTTTCGTACCAAAGATTTTGTCAC 3' (CDUPRT specific)
RevGFP3'primer: 5' AACCTGGAATTCGGTATACTTGTACAGCTCATCCAT 3' (GFP specific)

Primers designed to sequence pENTR.CDUPRT and pENTR.GFP constructs to confirm the integrity of the constructs:

FW-CDUPRT (primer for sequencing pENTR.CDUPRT): 5' TGCGCCAGAAGGTATCGCTGCG 3'
Rev-CDUPRT (primer for sequencing pENTR.CDUPRT): 5' CAGTTATGGGCATCACGCCGGA 3'

FW-GFP (primer for sequencing pENTR.GFP): 5' TGAGGCCCAGTTCTCCTCCC 3'
Rev-GFP (primer for sequencing pENTR.GFP): 5' TTGGCCAAGCATGGCAGCT 3'

Primers designed to authenticate and confirm the sequence of mIL-18 in pVITRO2-GFP/mIL-18

Forward primer mIL-18 –5' GTAAGAGGACTGGCTGTGAc 3'
Reverse primer mIL-18 – 5' CTCCATCTTGTTGTGTCCTG 3'